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PREFACE

This is the 25th anniversary of the *Annual Review of Biochemistry* and, according to the *Saturday Review*, the 2500th anniversary of the birth of Kohmar Pehriad (544–493 B.C.). This apocryphal and industrious scholar reputedly invented the comma and the small round dot, and his son Apos Trophe did exactly what his name suggests. At least so we are informed as, on April 1st, we prepare these paragraphs for the press.

It is time that all of us who have suffered under these inventions editors and authors alike endeavour to determine whether punctuated composition is worth its salt doubtless there are few whose reading of the literature goes back to the sixth century B C but there are many who have read with profit and with ease the free verse of archie the cockroach and mehitabel the cat as immortalized by don marquis in these volumes we have tended to minimize the comma and the hyphen neither of which is indispensable and each of which is a weariness of the flesh the period is something else again though had we the courage of omission we could cease as editors to trouble ourselves our authors and our readers with the alleged impropriety of the over long sentence with what a wealth of innuendo of ambiguity and of confusion our writing could be so effortlessly enriched that this is done at present is widely conceded but needless toil goes into the struggle to circumvent the watchful eye of editor and referee enough of kohmar pehriad and his dubious efforts to make reading easy.

The twenty-fifth anniversary of the *Annual Review of Biochemistry* is also year one of the *Annual Review of Entomology.* The introduction of this new Review must not be taken to mean that insects have ceased to interest the biochemist. It is rather a belated recognition of the fact that insect physiology and biochemistry have developed as a discipline of major importance and that insects now command, and deserve to command, the interest of the scientist almost as much as do the higher animals, the plants, and the microorganisms.

It is with deepest regret that we record the death of Sir Edward Mellanby and of Seymour Korkes. Sir Edward had entered upon the preparation of a preface, autobiographical in character, for this present volume. He died on January 30, 1955, and his colleague of many years, Dr. B. S. Platt, kindly agreed to write the preface which Sir Edward had planned and, indeed, commenced.

Seymour Korkes, a biochemist of notable achievement and of great promise, was killed in an automobile accident on December 10, 1955. At the time of his death he had made a substantial start on the review on Carbohydrate Metabolism. A number of his colleagues and intimate friends undertook the task of carrying the review to completion. Though the authors of this chapter are lost in anonymity the review will stand as a tribute to Seymour Korkes—his life and his work.

We are indebted to many who have made this volume possible, but especially to our authors whose task of reviewing the biochemical literature in a few hundred pages becomes more and more difficult with every passing year. We acknowledge gratefully the generous assistance of Dr. Donald Kupke and Carol Kupke in preparing the subject index. Upon Lillian Rutherford, our principal editorial assistant for the *Annual Review of Biochemistry*, fell the responsibility of assisting the Editors in preparing the volume for the press. Finally, we are indebted to our printers, the George Banta Company, Inc., for their patience and ever cordial co-operation.

F.W.A.	B.L.H.
F.S.D.	T.H.J.
H.J.D.	J.M.L.
W.Z.H.	G.M.

Harry Deuel
1897–1956

After this volume had gone to press we were advised of the death of our colleague, Harry Deuel, Jr. Dr. Deuel, long interested in the *Annual Review of Biochemistry*, had been a member of the Editorial Committee since 1946. In that year he was also elected to the Board of Directors of Annual Reviews, Inc., and served as its President since 1953. His distinguished contributions to biochemistry and nutrition, and his years of invaluable service to the University of Southern California as Professor of Biochemistry and as Dean of the Graduate School are widely known. To those whose good fortune it was to know him intimately he will long be remembered for his high personal integrity and for the real warmth of his friendship.

He died of cancer on April 17, 1956, carrying on to the end with tremendous courage and fortitude.

ERRATA

Volume 24

page 125, line 30: *delete;* contrast 177
page 219, lines 1 and 4: *for* citrus *read* fungal
page 230, line 26: *for* 12.1 kcal. *read* 12.1 entrophy units
page 265, reference 117: *for* 1954 *read* 1945
page 343, Table I: *for* 1. Vitamin (B_{12}) cyanocobalamin) *read* 1. Vitamin B_{12} (cyanocobalamin) and *for* 2. Vitamin B_{12} (hydroxo- or aquocobalamin) *read* Vitamin B_{12b} (hydroxo- or aquocobalamin)
page 623, reference 208: *for* Grove, J. P. *read* Grove, J. F.
page 624, reference 211: *for* Grove, J. P. *read* Grove, J. F.
page 645, line 8: *for* NCN *read* collagenous N

Author Index

page 754: *insert* Fried, R(ainer), 393–418; 401
page 754: *insert* Fried, R(udolf), 422, 423
page 754: *delete* Fried, R., 393–418; 401, 422, 423

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E. Mellanby

PREFATORY CHAPTER

Sir Edward Mellanby, G.B.E., K.C.B., M.D., F.R.C.P., F.R.S. (1884–1955): The Man, Research Worker, and Statesman

By B. S. Platt

Human Nutrition Research Unit, Medical Research Council Laboratories, Hampstead, and Department of Human Nutrition, London School of Hygiene and Tropical Medicine, London, England

Sir Edward Mellanby had accepted a cordial invitation from the Editorial Committee of the *Annual Review of Biochemistry* to contribute this Prefatory Chapter. Unhappily, before he had completed his seventy-first year, he died suddenly on Sunday, January 30, 1955, about mid-day, after he had spent the morning at his research. His draft of the introduction to his contribution to this volume is printed below in the section "Background to British Biochemistry."

For nearly six years after retiring from the post of Secretary of the Medical Research Council, he continued to engage in research work and in many scientific affairs. He was, for example, active in the Royal Society, of which he had been a Fellow for 30 years, and he was one of its Vice-Presidents. Sir Henry Dale has recently completed an Obituary Notice of Mellanby for the Royal Society (1955);[1] this tribute is of such grandeur as perhaps only a man of Sir Henry's eminence could contribute, and I shall quote from this to give the weight of wisdom, experience, and knowledge to this chapter.

It has been said that until the First World War we in Britain were apt to assume that if foreigners did not understand or even like us, it was their fault and not ours. Various British statesmen from time to time have questioned whether this *laissez-faire* attitude was reasonable or rewarding, but it was not until 21 years ago that a committee was set up with the title "The British Council for Relations with Other Countries." It is indicative of Mellanby's broad interests that he took an active part in the work of what is now known as the British Council, and he was Chairman of its Advisory Medical Panel from its inception in 1942. One of the projects he initiated shortly before his death was the preparation of a special number of the British Medical Bulletin (1956)[2] on recent advances in vitamins. This number is the poorer by the fact that he was unable himself to contribute on his work on vitamins A and D; it is, however, to be issued as a memorial to Sir Edward Mellanby, and it contains among other notices of his work, a personal appreciation of him by Sir Charles Harington. Other appreciations

[1] Dale, H. H., *Biographical Memoirs of Fellows of the Royal Society,* **1,** No. 24 (1955).

[2] *Brit. Med. Bull.,* **12,** No. 1 (1956).

appearing shortly after his death in the British medical and scientific press[3,4,5] all make my task, if anything, in some ways more difficult. I still feel, as I felt some months ago when preparing a notice, as Mellanby himself said he felt when faced with the preparation of the Hopkins Memorial Lecture (91), that the task requires a "degree of knowledge, skill, judgment and sympathy . . . almost unattainable."

Most readers will be familiar with much of Mellanby's scientific work, and they will know something too, no doubt, about the influence he wielded as Secretary of the Medical Research Council of Great Britain. Whilst both of these topics must be given consideration in the chapter, I feel it would be interpreting the wishes of the Editorial Committee if a glimpse is given of Sir Edward's life before we look at his work.

A Biographical Sketch

"Nobody" writes Sir Henry Dale[1]

> who had dealings with Edward Mellanby could fail to be attracted by his big, handsome, friendly personality, which, in his happier moods, had retained something of a boyish exuberance. His character was strong and independent, and, when he considered the opinions of others, he looked at them on their merits and with little reference to the standing or the dignity of their advocates. . . . He was a doughty champion of the interests of medical research as he saw them, and a man of great personal achievement in its performance; and, with it all he was a man of simple, generous and lovable character, who could be tender with his sympathy for a friend in trouble, as well as fierce and impetuous in his drive for what he believed to be right and important.

He was born on April 8, 1884, in West Hartlepool, the youngest of a family of six children and the last of four sons, one of whom died in childhood. John Mellanby, his father, was the manager of a shipyard in the Furness-Withy Company, and his mother was born in Edinburgh. Dale describes the parents as "members of a Free-Church communion and the children were brought up in an atmosphere of evangelical piety with austere standards no doubt, of conduct and enjoyment, but tempered and enlivened by a robust independence and a genuine regard for intellectual achievement and enterprise." Mellanby's father, a Yorkshireman, was noted for his management of the men in the shipyard and for his ability in handling people, and he no doubt passed on some of this knowledge of human nature to his sons when he enjoyed his evening stroll with them; he was at one time amateur boxing champion for the north of England and he taught his sons boxing. At Barnard Castle School Edward Mellanby excelled at sports; he won several jumping and running events and was captain of both cricket and football teams; he is described in the school magazine as "the best outside left we have had for some years." It was his reputation as an athlete that lived on to

[3] *Brit. Med. J.*, **I**, 355, 421 (1955).
[4] *Lancet*, **I**, 309, 359 (1955).
[5] *Nature*, **175**, 530 (1955).

impress Professor J. H. Burn of Oxford, when, as a new boy, he joined the school shortly after Mellanby left. At the Cambridge College sports in 1903, he won the Freshman's race of 200 yards, his time being $24\frac{3}{5}$ seconds. Unknown to many of his friends and acquaintances of later life his interest in sport continued. He used to watch the Sheffield football teams on Saturdays, and he would interrupt his work on Saturday afternoons at the Nutrition Building to hear the Test Match commentaries. If his team was not doing well he would turn to his assistant and say "Come on, they can't play cricket nowadays, let's get on with some work." No doubt his early athletic activities helped to lay the foundation for the almost uninterrupted good health he enjoyed throughout life. He took a cold bath every morning to the day of his death!

His prowess in athletics was matched by his scholastic ability. He won the Upper School prize and a special prize for theoretical and practical physics and, like his brother before him, he took a Leaving Exhibition from school to go to Cambridge; there being three or four years between each of the brothers leaving school, the Exhibition was "a Mellanby possession" over a number of years. John Mellanby preceded his brother Edward to Emmanuel College, Cambridge, and it is on record that it was the particular bottle of acetic acid allotted to John in Hopkins' advanced practical class which, by leading to an anomalous failure of the Adamkiewicz colour reaction[6] for proteins, played a more or less direct part in Hopkins' discovery of tryptophan. John Mellanby held the Chair of Physiology at Oxford at his death; the eldest brother was Professor of Civil and Mechanical Engineering at the Royal Technical College, Glasgow. Mellanby's sister describes Edward in his early days as very lively, as always being in evidence in the home since he was the youngest, and a mimic of all the visitors. Throughout his life he enjoyed a joke and fun, and he had in his nature a strong streak of mischievousness. His discovery that canine hysteria was the result of the use of agene in "improving" wheat flour he kept to himself for almost a year before he sprung the news on the Physiological Society with almost impish glee. He was what we call in the north of England a "tease."

He loved children, and they loved him; he did in fact enjoy young people of all ages, and he would make a special effort, a little sheepishly, to see a new baby. One day he appeared at my kitchen window in muddy boots carrying an enormous cabbage he had grown himself which he presented with a smile as a fitting tribute to a family of seven. In his later years he took an increasing interest in the garden around the Nutrition Building, and with some pride and manifest delight he exhibited a wide range of produce from these gardens and more or less swept the board of the prizes and trophies at a show of the gardening club of the National Institute for Medical Research. One of his treasures which he obviously enjoyed was the framed original of a

[6] This reaction depends on glyoxylic acid which may be an impurity in acetic acid (see 91).

cartoon[7] from a Glasgow newspaper published in 1922 poking fun at the professor's work on the rickets-producing action of oatmeal.

He was in many ways eminently a sociable person. His colleagues recall with pleasure sing-songs at the Mellanby home in Sheffield, his own contribution being made on a tin whistle. He had a pleasing voice both in speaking and in singing, and when experiments were going well his voice might be heard echoing down the corridors of the laboratory in song. He was genuinely musical, with an inclination towards Bach and away from Bartók. He had, a colleague writes, "a boyish, genial manner, an exuberant zest for life and a sense of humour which made him a very likeable and stimulating colleague and chief." It is regrettable that to some who did not know him well, Mellanby might sometimes have appeared perhaps somewhat aggressive or arrogant or over-critical of other people and their work.

A student, recalling memories of Mellanby as a professor 30 years ago, stresses how Mellanby had become to him:

> the prototype of the professor of real life instead of that odd creature, the professor of fiction. In no way absent minded, nor in the clouds, nor academic in the futile impractical way in which this word is now misused, he was large, healthy, robust, with a memory like a dictionary and a seemingly inexhaustible knowledge of physiology, chemistry, and biochemistry, not to mention pharmacology [of which he was professor]. And practical above all things.

Mellanby was in his later years much sought after as a lecturer, as the number of important named lectures in the appended bibiliography testify. His old student retains the impression that his professor in his lectures "took nothing for granted, but brought always a fresh outlook to everything which came to his notice. Never shall I forget" he writes "his lecture upon alcohol. 'Let us examine the facts which have been proved, whether we like alcohol or not' " opened an hour's undergraduate lecture which is still remembered clearly by his student who claims he has not heard anything better worth listening to since. As a young student of 23 years he was impressed with a number of incidents: Mellanby's remark on research and committees "If you want to find out anything, don't imagine you'll do it by talking about it at a committee meeting. You'll have to do some work on it, and hard work too"; his reply to a student who asked why a certain drug acted as it did, "science does not explain why: it tries to answer how"; and the only time when he was seen to be really annoyed, when a Scottish professor denied the results of Mellanby's work on the rachitogenic factor in oatmeal—he could have forgiven a different opinion, but "this creature had denied the facts." To the student waiting for examination Mellanby must have indeed seemed formidable; our informant recalls waiting his turn in the corridor of the department when the professor's wife passed by and evidently seeing signs of strain said "Are you going in for your viva? Oh, you'll be all right—he's really much nicer than he sometimes sounds."

I find myself reluctant to attempt to give a complete, detached, and

[7] This is reproduced as Figure 42 in *A Story of Nutritional Research* (96).

objective portrait; chiefly, I think, because to Mellanby I was—as many people even older than myself were—"young man." I can imagine him at this point coming out with some apt quotation, accurate and probably from the Scriptures, though it might equally be a line or two from a ditty of the music halls of decades ago, and his remark at this point might well be "Judge not that ye be not judged." Mellanby was a man for whom one could have the profoundest respect, admiration, and affection, yet he had a forthrightness of manner that alienated some, particularly those of a gentle or timid temperament. He demanded of others qualities which he himself possessed: wisdom, devotion to discovery, integrity of character, competence at work, and courage. He had little use for persons who did not have these qualities and often expressed his contempt for those who showed their antitheses—stupidity, indifference, untrustworthiness, and ineptitude. There were individuals he did not like, but he never allowed such dislikes to affect his decision to invite such a person to do a job if he judged him to be the right person for it. I know that it came as a surprise and sometimes as a shock to him to be accused of being deliberately unkind or unjust, when he had thought that he had been consistently helpful. The aggrieved person was often one who happened to be about, possibly failing to understand, or being out of sympathy with, some important principle Mellanby was out to establish, or some major measure which he was pushing through. His qualities as a man of affairs, in my view, are summed up in the term "statesman," and I have a letter from one the most eminent Civil Servants of our time and a man of exceptional judgment saying "I am glad you describe him as a statesman."

Two main streams are distinguishable in Mellanby's career, but just as in attempting to discover the origin of a river, so it is not entirely clear where Mellanby could be said to "rise," probably from a spring in Cambridge. His early days there are described by himself in the next section. The course of his life's work was, however, directly, if remotely, affected by the following programme drawn up in 1913 by the Medical Research Committee, forerunner of the present Medical Research Council:

> A list of major disabling diseases besetting the country at that time was prepared and those individuals who had shown a particular interest in any of these or were judged to be fitted for the task were asked to investigate one or more of the diseases from some specific angle. Among the items listed were tuberculosis, rheumatism (including acute rheumatic infections and chronic arthritis), rickets and oral sepsis (96).

According to the first report of the Medical Research Committee for 1914 to 1915 "Dr. Mellanby was given part-time work with an assistant supplied by the Committee for the study of experimental rickets and its relations to conditions of oxidation." Mellanby began this work whilst holding the Chair of Physiology at King's (now Queen Elizabeth) College for Women. The work was continued in Sheffield where he held the Chair of Pharmacology and a post as Honorary Physician to the Sheffield Royal Infirmary. This

post he prized because in addition to facilities for laboratory work he also had a number of patients in his care, and this gave him opportunities for extending his investigations and teaching into clinical fields.

In 1933 he was invited to become the Secretary of the Medical Research Council. His work in this position was not in fact a separate stream so much as a powerful current in the mainstream of his career, for his research continued throughout the 16 years that he was Secretary, and indeed after his retirement, until his death.

Mellanby and his work were well-known in many countries in the world, and his eminence was recognised by the conferment of Honorary Degrees by many Universities, of distinctions and prizes from many scientific and other bodies. "These awards and high honours" said Lord Hankey at the address given at the Memorial Service to Sir Edward Mellanby "add undying lustre to Mellanby's career and testify to the world-wide esteem in which he was held;" but he says "Mellanby won the greatest prize of his life by marrying May Tweedy, thus doubling his efficiency, for she was already a research scholar and lecturer at Bedford College and was ideally equipped to halve his troubles and double his joys."[8]

Background to British Biochemistry

The main part of this section is Sir Edward Mellanby's own introduction to the present prefatory chapter; it is reproduced substantially unaltered:

In writing the prefatory chapter to previous *Annual Reviews of Biochemistry*, my distinguished predecessors have discussed many of the salient developments in this subject during the present century in Germany and the United States. The invitation to write the present chapter can only be regarded as a gerontological distinction to one who has had the opportunity of seeing and appreciating the same kind of magnificent development of the subject in the United Kingdom, for the most part as a biochemist in the suspended chrysalis stage. Had fate been kinder, or at least different, the present writer might at least have been able to write with a little of the distinction of his predecessors; but whereas the trend of events has made the modern biochemist into a specialist chemist, those who entered the field in the earlier years of the development of the subject were more biological in outlook and their chemical skill and knowledge were elementary. The chemist of 1905 was not interested in biological phenomena, and he never for one moment considered it possible that the future of chemistry itself would be revolutionised by knowledge of the chemical processes which were encountered in biological material. It can be said with assurance, however, that those of us who were actively engaged in the study of physiological processes at that time realised to the full that the essential condition for progress was the enticement of first-class chemists into the biological field as active collaborators. Our prayers have been abundantly answered, indeed, more than answered, for the pendulum has swung the other way, and whereas chemical skill in biochemistry has become predominant, biological knowledge and outlook in the subject has either tended to fade away or to isolate itself from that of the chemists. This present-day dichotomy is

[8] Address given by The Rt. Hon. Lord Hankey at the Memorial Service to Sir Edward Mellanby, March 17, 1955.

viewed with regret by those, such as medical men, whose duty it is to deal with the practical problems of living. They realise to the full the great intellectual pleasure brought by discoveries of biochemists in, for instance, the subject of intermediate metabolism, and they appreciate greatly the advances in knowledge of carbohydrate metabolism, but they also know that they can do little more for their diabetic patients now than they could do 30 years ago, when insulin was first discovered by physiologists and the carbohydrate cycle was undreamt of. Nor can the gouty patient be greatly assured when he is told he is suffering from a biochemical disorder and that biochemistry is the most successful of all modern scientific subjects. There is much to be said for the view that biochemistry is a discipline in its own right, but there is still a great need for workers in the subject to direct their attention to the great practical problems of biological function. In such matters it is just as likely that the scientist will make a great scientific discovery by a study of the practical problem as it is for him to make a great practical discovery by studying a purely scientific problem; indeed, experience has shown that it is more so. Whereas in Germany and France it would probably be claimed that biochemistry had its origin predominantly in chemistry, in the United Kingdom there is no doubt that physiology was its progenitor. Thus in Germany, Liebig and his school; and in France, Pasteur and the pharmaceutical chemists, were outstanding leaders in chemistry who applied their great skill to problems of biological interest. In England we had no such distinguished scientists turning their attention in this way. Indeed, where such a person appeared, as in the case of MacMunn who made many fundamental discoveries in the chemistry of haemoglobin and blood pigments, his work passed unnoticed for 50 years or more, largely, it is presumed, because there was nobody in the country interested in this aspect of biological chemistry.

In the United Kingdom the man who did most for the official recognition of biochemistry as a discipline in its own right was Benjamin Moore. The first chair of biochemistry in England was founded by Liverpool University (the Johnston Chair of Biochemistry) in 1902, and Moore was the first incumbent. In 1920 the Chair of Biochemistry was founded in Oxford University by a Mr. Whitley, an intimate friend and former colleague of Moore's, and Moore was again the first professor. His early death in 1922 was followed by the election of R. A. Peters (now Sir Rudolph Peters), a pupil of Hopkins, as professor of biochemistry. An even greater service to biochemistry in England was the foundation in 1906 of the *Biochemical Journal* by B. Moore in co-operation with Edward Whitley. The journal was edited by Moore until 1912, when its control was taken over by the Biochemical Society. However, the real stimulus to British biochemistry came from Cambridge University, and in particular from its physiological department. Michael Foster had been imported to Cambridge by T. H. Huxley and ultimately became the first professor of physiology. As is now well known, Foster himself was no great scientific discoverer, but he had many other qualities of greatness, particularly those of appreciating the value of experimental investigation and, what is of greater importance, an eye for picking men with outstanding qualities in research and persuading them to devote their life to their studies. When I went to Cambridge in 1902, Foster had just retired and devoted all his time to parliamentary work (and to his garden). Langley had succeeded him as professor, and the small laboratory was filled with men of the highest distinction—these included Gaskell, W. B. Hardy, H. K. Anderson, Keith Lucas, T. R. Elliott, J. Barcroft and most important from the present point of view, F. Gowland Hopkins. It was a wonderful galaxy of talent, and any young man who had the privilege of entering and

being accepted by this circle was thereafter doomed to a career of research and investigation.

Hopkins was not originally a Cambridge man, and his importation at the age of 40 years, on the basis of a few publications of work done at Guy's Hospital, was a stroke of genius and one of the most fruitful incidents that can ever have happened in the world of science. Hopkins was made reader in physiology and was also given a post as director of studies in medicine at Emmanuel College. Entering that College in 1902 as a medical student, it was my great fortune to be brought at once under his scientific care, a relationship which continued first as student, later as research associate until 1907, and until his death in 1947 as close friend. After I had taken my bachelor's degree in 1905, Hopkins invited me to continue as a research worker in his laboratory. He had at this time completed his work on the detection and isolation of trytophan and was engaged, in association with Edith Willock, in experiments on growing mice to see what special part this substance played in animal nutrition. It was this work on tryptophan metabolism, published in 1906, which opened up the whole field of differences in protein quality, and so replaced the view that protein in itself was a nutritional entity; it also directed Hopkins' attention to the physiological importance of accessory food factors in general, which led to his classical publication on this subject in 1912. I remember with great interest the remark he made on a number of occasions in those early years that "Somebody was soon going to make an enormous fundamental discovery in nutrition." Little did he seem to recognise that, with his work on the nutritional needs of tryptophan and of other "accessory food factors," he himself had already made the necessary fundamental observations which were going to transform biological science and indeed to lead to a new science affecting the nutritional condition of the whole world. If anybody doubts this statement, let him read a physiological text-book on nutrition published before 1907.

After 1907 it was never my good fortune to work with Hopkins again, as I left Cambridge in order to become medically qualified. In 1920, when he became the first professor of biochemistry at Cambridge and obtained a laboratory which was worthy of him, he invited me to become his first reader, but by this time I had become so involved in medical research as the result of some investigations on rickets which he had urged me to undertake on behalf of the Medical Research Council (at that time Committee) that I decided not to return to Cambridge. This was a crucial decision (and probably a wrong one), and hereafter I could no longer be regarded as a biochemist. Indeed, my occupation was now as physician to a general hospital and as professor of pharmacology at Sheffield University—a subject which at that time did not make large teaching demands and so allowed time for investigation.

Up to 1914 Hopkins had to do his work in the Physiology Laboratory under what would now be regarded as ridiculous restrictions as regards space and laboratory assistance. He had one small room in which to carry out his investigations, and this I shared with him during 1906 and 1907. I remember with interest that all our clean glass (cleaned by ourselves) had to be kept in one cupboard, which remained locked to prevent raiding by fellow workers. In order to obtain any glass article from the permanently locked cupboard, it was necessary to remove an upper drawer and dig in the darkness for the particular flask or beaker required. In 1914 the physiology department obtained a fine new laboratory. Hopkins was raised to the status of professor of biochemistry and was allowed to use the whole of the old physiology laboratory. After the first war, biochemistry was recognised as a separate subject for Part II of the Natural Sciences Tripos, and in 1925 Hopkins for the first time was provided with an excellent institute for biochemistry, and it became possible for him to

receive into his laboratory a group of research workers who formed a good nucleus for the training of many of the biochemists who now form a significant part of the leaders of this subject in the United Kingdom. By 1925 Hopkins was 64 years of age, but fortunately his Chair was founded before the University rule of retirement at 65, so that he got the opportunity not only of extending his own great investigations but also of building up his well-known school and exerting his inspiring influence on biochemistry until he retired from the chair in 1943.

It would be impossible to exaggerate the magnificent part played by Hopkins not only in the development of biochemistry in England but also in setting a standard of investigation which very few can attain or even copy.

In 1948 Mellanby delivered before the Chemical Society the Hopkins Memorial Lecture. In it he relates an incident which is an interesting footnote to his appreciation of Hopkins. He said

Hopkins had a great stimulating power on young people. A particular instance of this kind happened at an early stage in my undergraduate career at Cambridge, when he introduced to a class taking the Tripos in Physiology a discussion on the volume of blood in the body. He succeeded in rousing in me a state of enthusiasm sufficient to make me confine my whole attention to this problem for a fortnight to the detriment of my other work in order to find a method to decide whether the blood volume was 1/13 or 1/20 of the total body weight. This was an exaggerated reaction of the kind with which I was constantly affected in the few years in which I was intimately associated with him in the laboratory.

An Autobiographal Sketch of Research

The Editorial Board emphasise the great value of these prefatory chapters to the younger generation of biochemists and especially of the autobiographical sketch of the life of the person as student, teacher, and investigator. Mellanby, in his Abraham Flexner Lectures which have been published under the title of *A Story of Nutritional Research. The Effect of Some Dietary Factors on Bones and the Nervous System*, has already given an autobiographical account (96) of "a series of investigations which systematically developed from observations made early in his career, observations which played a prominent part in the birth of the science of nutrition and which have continued on and off over a period of thirty years." The "story" runs to well over 100,000 words with many diagrams and tables; to summarise it would defeat the purpose of the story. Sir Edward intended it to be read through and to be a source of inspiration; as a demonstration of the triumph of determination over the difficulties of biological research, it will undoubtedly become a classic in the literature of science.

Rickets was, as already mentioned, the subject allotted to Mellanby by the Medical Research Committee in the days immediately before the First World War. The state of knowlege of the disease at that time is indicated by the nature of this assignment, which was to investigate the part that processes of oxidation might play in the aetiology of the disease, and, in the early stages of the experiments which began in 1915, tests were made with diets of high specific dynamic action to see whether increasing the intensity

of the oxidation by this means affected bone formation. In 1919 to 1920 it was demonstrated that a fat-soluble vitamin controlled calcification of bones and teeth. In course of time it became evident that the vitamin A of those days was a complex of two vitamins, one retaining the name of vitamin A and the other now called vitamin D. In his Flexner Lectures, Mellanby showed how

> these two entities work together in close association. First vitamin A controls, or at least influences, the activity of the osteoblasts which lay down the soft bone matrix. Vitamin D then attends to the calcification of this osteoid tissue, and finally vitamin A again steps in and sees that any superfluous calcified bone is removed by osteoclastic action and that the bone shape is correct. Thus . . . these two inseparables, vitamins A and D, the David and Jonathan of nutrition, whose faithful alliance in distribution and similarity of many chemical and physical properties has caused so much trouble to hosts of physiologists, biochemists and other scientists, work in harmony and on the same structures at the time of their active careers in the animal body. Although their functions are different, they unite in directing and controlling the building up and maintenance of bone structure.

With these words Mellanby concludes his account of a series of researches. Selecting from Chapter X of this book an illustration of his treatment we read that he sets out "to find the cause of the ataxia and incoordination of movement that developed in growing animals fed on diets, which so far as was known were complete except for vitamin A and carotene." In the earlier chapters of the book he has described "the development of the research and the gradual unravelling of the factors involved as the work progressed." Whilst from this work, "it is probably easy to judge" he says, "where and when progress was made and generally to group and evaluate the positive results, it cannot be easy to understand the doubts, difficulties, disappointment and mistakes that have been major factors in work which has continued intermittently over a period of about 25 years."

He refers

> to a brief account of experiments which failed to open up the problem, but the narrative would hardly bear a full review of all the other will-o'-the-wisps that were pursued in the course of the work. Yet, failure fills a much larger part in biological investigation of this nature than in the more scientifically developed chemical and physical fields. The solution of a biological problem when worked out often seems so reasonable and so simple that wonder is expressed that any other sequence of events could be contemplated, but those who think along these lines can have had little experience in opening up new fields of biological enquiry. Such work involves constant speculation and formulation of ideas, most of which, on testing, do not reach the stage of being acceptable as working hypotheses. It is even worse when the speculation becomes a working hypothesis only to be rejected after months or years of further work.
>
> One other general remark may be permitted. It is obvious that there is no end to an investigation of this nature. The final results [as he has described them] will no doubt form the basis for other work. The facts as described will probably be for the most part, correct, but it is rare for biological discoveries to be interpreted in the right light

or in their true perspective in the early stages. There is no reason to believe that the present work will differ in this respect from many other advances in biological knowledge.

After summarising the changes attributable to the presence or absence of vitamin A in growing bone, demonstrating so clearly how important a task vitamin A has in ensuring the normal moulding and shaping of bones, he writes

It will be generally agreed that Nature has done well to provide the central nervous system with a strong bony protection. Its safety from assault is essential both for the survival of individual and the race. Those who build the walls and ramparts must, however, plan their activities in accordance with the size and growth of the citadel to be protected. The central nervous system is a citadel and as it grows the protecting walls are normally moved farther out. As the bones of the skull and vertebrae grow bigger the space they surround is enlarged by absorption of the inner surface and deposition on their outer surface. Now if the director (vitamin A) of building operations disappears, it might then be expected that the brick layers (osteoblasts) and demolition squad (osteoclasts) would either go on strike or work in a completely disorderly way, but this does not happen in the bony ramparts. It is rather as if the place of vitamin A as a wise director of operations were taken over by that worst kind of director—the energetic man with no wisdom, whom we all know so well nowadays—who says: "I am going to show you how things should be done; now you will see something really happen." His directions are: "You must work harder than ever, but in a different way. You builders (osteoblasts) must lay down bricks wherever there is a foundation (periosteum). You demolishers (osteoclasts), working nearest the citadel, must leave that position and continue your labours elsewhere."

The result is that the walls, instead of enclosing a greater area as the citadel grows, now encroach on the nerve control stations, lines of communication and the administrative centres and squeeze all the vital structures into so small a space that work inside the citadel is impossible. Parts of the citadel (the central nervous system) are destroyed and the city (animal) with it. There has been no slacking and no anarchy among the building operatives but, by working at the wrong place and in the wrong direction, they have converted a protective structure into one of destruction. Thus, vitamin A, by regulating the activities of the bone cells in this position coördinates a beautiful adjustment of bone and nervous system growth. How important this function is to animal development can be appreciated by the drastic and dramatic effects produced when the mechanism goes wrong in the absence of the vitamin.

The second half of *A Story of Nutritional Research* is devoted to a study of the anti-calcifying or rachitogenic action of cereals. Mellanby starts at the point where rickets is accepted as being a result primarily of a deficiency of the vitamin D moiety whose main function is to harden the bones by controlling the deposition of the calcium-phosphate compound in the osteoid tissue laid down by the osteoblasts. He points out that

Prior to the Great War of 1914–18 . . . among a vast number of other hypotheses it was stated by clinicians that carbohydrate-containing foods, including cereals, had a rickets-producing effect on children. This view was largely founded on the fact that excessive consumption of such foods produced fat babies and in those days fat babies

were more prone to develop the disease. Rickets was at that time rampant in both the U.S.A. and Britain and the indictment of this large class of foodstuffs by clinical experts was widely accepted . . . while their assumption had real foundation, the explanation of the rachitogenic effect of cereals was not so simple as it then appeared, namely that the carbohydrate was the responsible factor.

He found two main effects of cereals, one on growth promotion which has not been elucidated and is, he says

undoubtedly . . . a subject which will repay further study.

The second one, to which most attention is given, is the property of interfering with the calcification of bones of young animals. Ultimately it was demonstrated that a chemical substance, phytic acid, plentiful in some cereals such as oatmeal, corn, and wholemeal wheaten flour, and sparse in others such as rice and wheat flour, has the property of interfering with calcification of growing bones and teeth. This substance interferes

with calcium metabolism when there is a deficiency of vitamin D, causes the production of rickets in young growing animals and, in adult animals, a decalcifying effect which may lead to osteomalacia . . . phytic acid or inositol hexaphosphoric acid, [is] a compound which has long been known to exist in cereal grains and, indeed, in all seeds in the form of the insoluble Ca and Mg salt—phytin. Phytin itself is not rickets-producing but oats and almost certainly other grains contain phytic acid which is not wholly satisfied by Ca and it is this form which has the toxic anti-calcifying action. It was found early in the work that the phytate action could be largely antagonised by the addition of extra calcium to the diet, especially in the first weeks of the experiment, while the presence of vitamin D completely preventeo rickets, although as shown later it did not necessarily prevent the predatory effect of phytate on calcium . . . more recent experiments have consisted of metabolic studies of the close interaction of three substances which are themselves, or which contain, elements essential for calcium metabolism, namely vitamin D, Ca and phytate, a rich source of P. If nature had supplied more phosphorus as inorganic phosphate and less in the form of phytate, the investigation would have been unnecessary. As it is, phytate provides much of the P essential to animals and its unhappy property of combining with Ca in the intestine and so making this salt unavailable to the animal is the cause of much physical abnormality. It would be wrong however, especially from a scientific angle, if all interest in phytate as a physiological study centred round its property of immobilising Ca in the gut. As a rich source of inositol, an essential dietary constituent in many animals, and a well recognised constituent of nervous and muscle tissue, phytate is clearly of some importance from another angle.

It is impossible to study the calcification of bones in growing animals without being impressed by the dominant position held by vitamin D. Take all vitamin D away from the food and ultimately, when the reserves of this substance are lost, the animal cannot retain sufficient Ca, however much is added to the food . . . when what appears to be a relatively small amount of this vitamin is supplied to the body further additions do not increase the powers of the body to absorb and retain calcium. This does not mean that the additions have no effect on the bones, for it was shown that increasing the vitamin intake resulted in an improvement in bone quality even when it had no effect on calcium absorption. It does this by controlling the proportion of organic to inorganic substances.

Although vitamin D has been shown to stimulate "the absorption of Ca from and hydrolysis of phytate in the gut," it seems certain

> that this does not explain all the action of the vitamin, and that it also plays an important part in promoting the deposition of the calcium-phosphorus compound in the osteoid tissue of the growing bone and in producing perfect bone structure.

Mellanby concludes his "story" by discussing some of the evidences of

> the present improvement in calcification processes in the population of Britain during and since the war . . . in spite of the greater relative consumption of [cereals], involving as it has, the higher intake of phytate. Here, then, is some evidence of a practical nature, taken from the life of the community, showing that the teachings based on the results of animal experiments . . . are correct and that cereals with high phytate content, although in themselves harmful and even dangerous to life, and especially early life, can be made innocuous by including in the diet other foods rich in calcium and containing vitamin D.

Half a Century of Research

In 1905 Mellanby started research in Gowland Hopkins' laboratory; he worked for two years on creatine and creatinine metabolism. In a paper published in 1908, evidence was presented which decisively disposed of a number of current notions about the behaviour of these substances in the body. The first signs of his life-long interest in biological problems are to be found in this paper; he examined the creatine content of various animals, and its occurrence in the course of development in several of these, in an attempt to discover whether from ontogeny and phylogeny light might be cast on the evolution of vertebrate animals. A bibliography of Mellanby's work is appended, with titles, so that the range and scope of his work can be appreciated. An attempt will be made here to indicate how the various researches may be linked.

The sorts of strands of thought running through much of his research are best appreciated from the work described by Mellanby himself in *A Story of Nutritional Research*. One feature is the way he follows up one piece of work with another. In his earlier papers for example he returned from time to time to the study of creatine in metabolic processes; this is to be seen in a paper on the metabolism of lactating women which Gowland Hopkins read before the Royal Society in 1913. An incidental observation that creatine is destroyed by bacterial action led to a study, with F. W. Twort, of the effect of bacteria from the gut on creatine; this work led to the discovery that histidine was decarboxylated by an intestinal organism to form histamine. These threads are picked up again in a paper on the investigation of diarrhoea and vomiting of children.

Another theme of considerable importance is what he calls "the interaction of clinical and experimental work" which he used as a sub-title to his book *Nutrition and Disease* (58) published in 1934. He says in the introduction to this book

> Some lay stress on the importance of the clinical aspect of medical science, others

on the provision of laboratory facilities. Only too often there is a distinct cleavage between the respective adherents of these two schools of thought.

His book is offered as "a chance, by reviewing the work done, of showing how the two methods can react on one another to their mutual advantage."

Some of his work seems to derive from his desire to maintain a sense of balance, a reflection of his sense of propriety and level-headedness; this emerges, for example, in his chapter on "Toxamins in Food" contributed to *Perspectives in Biochemistry* (67). He opens the chapter with this paragraph:

> It is curious how biological research is influenced by vogues and fashions: probably not to the extent of dress, but still greatly. Nor is this altogether a bad thing. It means that a profitable idea is often pressed until it looks like a squeezed lemon, but, unlike the lemon, it is not really dry and is only put aside and not thrown away. After a moribund period the original line of investigation is taken up from another angle, the old fashion is revived, and all the workers in the subject are after it again.

Remarking that this is a phenomenon particularly apparent in nutritional investigations he refers to the fact that Chatin in 1850 put forward the first instance of deficiency disease when he ascribed simple goitre to deficient intake of iodine in food. This ". . . excellent work" he points out, "was discredited, partly by the French Académie des Sciences, and partly by the success of the Pasteur investigations on microorganisms which led the medical world to believe that all disease is due to a *materies morbi,*" only to be revived later with Baumann's discovery of iodine in the thyroid gland. Mellanby gives evidence to show how in the first part of the present century

> . . . the pendulum swung towards the deficiency theory of nutritional disease with a vengeance, and well has the theory served its purpose. The object of the present contribution, however, is to swing the pendulum back a bit and emphasise the view that some so-called deficiency diseases are not simply due to the deficient intake of dietetic entities but depend also on the action of certain positive toxic factors which are normally present in food (67).

Another powerful influence in his work was the practical and humanitarian benefits that he recognised might accrue from medical research. His investigations in the earlier part of his career into the physiological action of alcohol, for example, did much to influence opinion and policy in regard to the social evil of drunkenness in this country. He took an active part in one of the earliest campaigns on cancer research and himself engaged in investigations into the cancer problem in which he was still interested in his last years, as a quotation from an unpublished report given later will show. It is not surprising that working in the district in which "Derbyshire neck" (goitre) was common, he should engage in work on this subject, and here again his interest continued as will be seen from recent publications with Dr. Fell on the effects of substances from the thyroid gland on living tissues cultured *in vitro.*

In a Presidential address to the Indian Pharmaceutical Association (100) he recalls that when a

. . . medical student doing clinical work in the years 1907–10, I constantly wondered at the attitude of my clinical teachers towards individual cases in hospital. Infinite pains were taken to diagnose the illness, and teaching at the bedside of diagnosis was very thorough. This was excellent. But after the diagnosis was made, I looked forward to learning the proper treatment of the condition. To my surprise, the physician either passed on to the next case without mentioning the subject, or in a perfunctory manner issued a few orders to the house physician and left the case. There was no teaching on what I thought ought to have been the main object of the physician, namely, to cure the patient. Only slowly did I understand this method of procedure, but looking back it is clear that his omission to discuss treatment was only too often due to the fact that there was no adequate treatment for these sick people. In many cases the physician said with a smile that this was a case for "expectant treatment" —clearly an expression of the hope that having diagnosed the complaint, Nature would play her part and cure the patient. Nature fortunately often rose to the challenge and cured the patient but this was by no means always the case.

Mellanby was one of the first persons in this country successfully to employ insulin.

An interest in infections and the possible role of good feeding in combating them constantly recurred. One of his most recent statements (86) on this subject reads

Nothing seems to be more certain than that nutritional defect is at least part of the aetiological basis of many of the more common illnesses, and yet, in most of these cases, this is only conviction without satisfactory proof. . . . To what extent, if any, dietary factors will be found to have a specific anti-infective action remains for future investigation. Some investigators on nutrition have already had experience in the search for this El Dorado, and in consequence regard the problem with more respect.

One of the best known of Mellanby's discoveries is that agenised wheat flour causes "canine hysteria" and that the toxic substance, methionine sulphoximine, produced by the action of nitrogen trichloride on gluten, has toxic effects on the central nervous systems of all the species of animals tested. This work has contributed to the present day interest in the effects on human health of chemical additives deliberately introduced into or present unintentionally in foods. Mellanby, in a lecture on "The chemical manipulation of food" (99), has sounded a warning against the dangers of leaving the field of food technology entirely in the hands of the "food scientist"; the "actions and reactions of the human body are so unpredictable, and knowledge of them so meagre, that the confidence and clear-cut views of the chemists may often be dangerous." One of the conclusions of his survey of the use of chemicals in the preparation of foods emphasises this warning.

The triumph of medical science in the prevention and control of disease during the present century has been impressive, and care must be taken that, at the time of gaining such control, new habits of living which cause ill-health should either be prevented or, if they arise, be controlled. Such errors in living are undoubtedly developing at the present time, and it is the duty of medical science to find out their relative disease-producing importance. The chemical manipulation of food may well be the basis of some of these errors, and the problem requires investigation (99).

The bulk of his work was, however, more or less directly related to his first important problem, i.e., rickets. This is true, of course, of all his work on bones, of the action of vitamins A and D, and work on toxamins, especially phytate; of his interest in diseases of the nervous system; of his experimental work on vitamin A and infections; and even the work on thyroid arose out of a similarity in histological appearance that he and his wife had observed between the hyperplastic thyroids of dogs under experiment and those of patients suffering from exophthalmic goitre (19).

The culmination of Mellanby's work on vitamin A came after he had delivered the Abraham Flexner Lectures. This work, which was part of a plan to examine the direct effect of substances on tissues in culture, he developed with the expert help of Dr. Honor Fell of the Strangeways Research Laboratories, Cambridge. They found that bone grown in plasma with a high vitamin A content showed some striking changes.

> The matrix of the cartilage first lost its metachromatic staining properties and then rapidly disappeared, leaving the healthy cartilage cells collected together like a bunch of grapes. The bone shaft then began to shrink and to disappear so that at the end of ten days or so nothing remained but a few crumbs of debris scattered in a sheet of actively growing and migrating cells.[9]

Chick ectoderm grown in plasma with a high vitamin A content developed not into normal skin, but into more highly developed mucous-secreting ciliated respiratory epithelium, and, when returned to normal plasma, it reverted to normal skin. Likewise, on culture in high vitamin A plasma, corneal epithelium became hyperplastic like the conjunctiva. Commenting on these results on metaplasia of epithelium, Mellanby writes

> The action common to skin and bone seems to be, therefore, that the effect of the vitamin A is on the basal structural cells both of the ectodermal and mesodermal tissues. If this is the right interpretation, vitamin A might be regarded as a "director" of basal cell development. This recalls the action of "organisers" in embryological growth. . . . It may well be that the developmental and differentiation changes of all organs, tissues and cells have their specific chemical controllers. In the case of cells, these controllers may normally continue to exert their influence over structure and function throughout life, and, only with their reduction or disappearance due to age or other circumstances, the cells may suffer metaplastic and degenerative changes.

He envisages the possibility that his results

> may ultimately prove to have a bearing on pathology and medicine. . . . The most prominent medical problem of metaplasia is seen in cancer, and it would certainly be beneficial to the scientific advance of studies in cancer if the biological mode of control of cell structure were understood. . . . The fact that various levels of a substance normally circulating in the blood and essential to life can have such remarkably different effects on the structure of living cells of different origins, and that these or some of them, can be analysed by a simple *in vitro* method, opens up the field of metaplasia to further study.
>
> Apart from metaplastic disease, it is possible that other diseases which involve degenerative and other changes of structure due to altered metabolism may be investigated by this type of work.[9]

At the time of his death, he was investigating with the help of colleagues in other Medical Research Council laboratories the effects of vitamin A on the metabolism of the sulphur-containing elements of skin epithelium, cartilage and bone[9]. Summing up in 1953 his attitude to his own research[10] he wrote:

. . . there is a tendency in work of the kind done in this [his own] laboratory to allow its chemical and biochemical aspects to become dominant. This I have resisted, and although chemistry has had to have its place, I have endeavoured to make it subsidiary to the biological objectives and to limit its expansion. It seemed to me that while the physiological field in its wider aspects is probably not receiving sufficient attention in this country, biochemistry on a large scale is developing rapidly into a special branch of chemistry and only too rarely with any direct relation to biological function. Thus, at the present time a large number of chemical substances have been isolated from living tissues, which are known to be essential to life yet there is little or no knowledge of the part payed by them in the animal economy. A knowledge of their functions might revolutionize our present-day physiology and pathology.

Statesmanship

It is most regrettable that Sir Edward Mellanby as one of the "elder statesmen" of medical research could not himself have recorded in this section his advice and experience. To his work as Secretary of the Medical Research Council he brought the qualities of a statesman—sagacity, far-sightedness, and skill in the management of practical affairs—in unusual measure. In a Minute of the Council on the occasion of his death, it is recorded that he

. . . took the leading part in the difficult tasks of deploying the Council's scientific resources in support of the national effort during the Second World War and of reconstructing and notably expanding the organisation thereafter. To these responsibilities he brought an intense regard for scientific truth, a wide knowledge and deep understanding of medical problems, a sure sense of what was important in research, and a constant desire to encourage all who showed ability as investigators and ideas likely to lead to real discoveries. His endeavours had great success.

We have already seen that he had been in close contact with work of the original Medical Research Committee in, for example, his researches on rickets and also on alcohol. He was indeed in many ways intimately connected with the activities of the Medical Research Committee from its earliest days. In a letter written to Mellanby in August 1913, Hopkins wrote

My job is to research after researchers. The new [Medical Research] Committee is going to be no joke. Indeed I want to ask you the biggest favour I ever asked of you

[9] Lady Mellanby tells me that although this statement is correct, his chief interest was in his experiments on methionine sulphoximine, the effects of which he was studying by means of injections into fertile eggs and by tissue culture. He was, she says, thrilled with the results so far achieved, and he was proposing to extend the work and to include tests on cancer growth.

Some observations on the effects of sulphoximine on the growth of bones in tissue culture, on the development of fowl from injected eggs, and on cancer growth are being prepared for publication posthumously.

[10] Unpublished report (Medical Research Council) by E. Mellanby (1953).

. . . it is no less than this that I propose to ask—I wouldn't ask it only it is a job which you will almost have to do yourself. I want you to try and appraise the current chemical work in physiology, pathology and pharmacology. . . . If by the end of the first week in October you could prepare some sort of document pointing out the main lines on which chemical research is likely to help practical medicine you would be doing much for me and for chemical research. . . . What I am asking is really rather a big thing but I feel that the matter is important. What we do during the next year may determine the fate of that £60,000 [the Treasury grant] for a long time to come. To make a strong case, I want a young and alert mind to help my rusty one and it is to you alone that I can turn.

A letter dated October, 1931, to Sir Walter Fletcher, the Secretary of the Council, shows Mellanby's continuing interest in the work of the Council of which by that time he was a member. Fletcher had evidently asked him about a committee to look into the possibility of advancing the application of biochemistry to medical problems. Mellanby writes

The lack of status of the biochemist in the average hospital is only an instance of the bigger problem of which you are fully aware, namely, the small recognition given to any scientific work inside the hospital. The status of the pathologist and bacteriologist inside the hospital is generally not much better than that of the biochemist although individuals have by their personality or by their research sometimes obtained well-recognised positions. The biochemists are not free from blame as regards their lower status than the pathologists and bacteriologists; so many of them refuse to get a medical qualification or even to study physiology that the choice to fill hospital posts is lamentably small. When qualified they often are attracted to the flesh pots thought to be associated with private practice. Scientific medicine has a long furrow to hoe before it arrives; that it will do so I have no doubt at all. It must however justify itself and the quicker it does this the more quickly it will be recognised. The most certain method it can adopt to do this is by making valuable discoveries and making itself pre-eminent. Able biochemists must get medically qualified and meet the clinician on equal terms even on his own line. He has a virgin field for discovery and ought to recognise that a biochemical discovery in a clinical subject has the great advantage not only of shedding light on the diagnosis and treatment of disease, but also opening up new academic lines of investigation. I am, as you know, a blatant advocate of medical research for this reason, but except for yourself there is almost a complete absence of such recognition even among scientific men.

It would have been interesting to read why Mellanby accepted the invitation to become the chief executive officer of medical research in this country. At the time he was appointed he was enjoying good facilities for research, and there were assured prospects for him of a more important post affording still better opportunities for research. There may have been a number of reasons for his acceptance, but it does seem likely that he took the job because he knew what he wanted to do and because he thought he could do it well. As his experiments on rickets influenced the course of his researches, so his experience with the study of the disease and of the application of the results of research in eradicating it, may well have helped to determine his decision to engage in administration.

When he started work on rickets, there were many and varied views on its aetiology. Among these was indeed good evidence of the efficacy of cod

liver oil and butter fat as therapeutic agents, as will be seen in the lecture on rickets by Cheadle (1906)[11] who emphasises that "the only constant factor, always present, is the food factor." Notwithstanding such teaching, however, in the 1910's "a concoction called Marylebone Cream, an emulsion of linseed oil, was being distributed in London welfare centers for the cure of rickets" (53). Soon after Mellanby had established that rickets was a dietary deficiency disease, a revolution occurred. In London over three quarters of the school children had been found to have some signs of rickets (14), but when it became necessary in the early 1930's to determine the effects of calciferol on the disease in the human, no case of rickets could be found in the London clinics that had not already been prescribed proper treatment; the investigators had to go to a depressed area in the north of England to find cases on which to conduct their trials. No wonder Mellanby subscribed so strongly to the view expressed by Lord Moulton when he wrote (61) "Where we are reduced to observation, science crawls. Where and in proportion as you can use experiment, science advances rapidly."

The main function of the Medical Research Council has been defined as the promotion of "scientific investigations for the acquisition of knowledge likely to be of value for the prevention, diagnosis and treatment of disease, and for the maintenance of normal health and full human efficiency."[12] In his Harveian Oration in 1938 (72) Mellanby showed how state control of medical research can be compatible with freedom to the investigator.

> In this country, and even more so in the United States of America, scientific research is not supported by the public, even the enlightened public, on intellectual grounds but because it has been found to deliver the goods often enough to deserve special encouragements. . . . Wise men, both in public life and in industry, saw that scientific research was the life blood of modern existence—a means of providing easily the needs of mankind and the knowledge necessary for healthy existence under modern conditions . . . it was good business to throw a sprat of expenditure to catch a mackerel of large profit.

Into this background of realism he fitted his concept of how research should be developed

He repeatedly insisted on the importance of the use of the experimental method, which he has described as "the most important discovery that the human mind has yet used and developed." He maintained that Claude Bernard's point of view was the correct one, that is, that all experimental medicine was applied physiology. But, he says,

> . . . it is no more unlikely that discoveries of first-class scientific interest will result from work directed to the solution of practical problems of disease than that discoveries of first-class interest to medicine will result from the study of academic physiological problems (61). Every man working on a problem from either angle ought to be familiar with the other aspect (79).

[11] Cheadle, W. B., *Artificial Feeding and Food Disorders in Infants*, 6th ed. (Smith, Elder & Co., London, England, 274 pp., 1906).

[12] Report of the Medical Research Council for the Years 1945–48 (His Majesty's Stationery Office, London, England, 1949).

Once when asked what the Council's policy was in financing research workers he said (72) they had none except "to find the right men and back them in every possible way." He recognised that "the limiting factor in this progress (of medical discovery) will be the men of genius to make the first-class discoveries." He had described the right men as

. . . limited in number and cannot be appreciably increased by any known method. They must in general be what is known as clever men, but cleverness alone accounts for little. In addition they must be men of strong character, capable of hard and often disheartening work, with power to distinguish truth from falsehood, prepared to spend their lives with limited social contacts and to forego riches.

One might add that this is something of a self-portrait. Such men he recognised are rare and he considered that one aspect of the Council's work was, short of this ideal

. . . to attract able men, and by providing financial assistance and ensuring the proper kind of training, helping them to a career of medical research.

Although the discovery of suitable investigators was a limiting factor, the difficulty he foresaw was not that of obtaining knowledge but that of its application to human needs.

In nutritional matters, in a paper in 1927 on the duties of the state in relation to the nation's food supply, he foresaw a number of what have been comparatively recent developments in applied nutrition. On one point he stands firm: "no nutritional policy adopted by governments can be wrong if it places the health and the needs of the community as its first and guiding principle."

In the broader field of human welfare he saw that

For every problem—social and economic—raised [in his Rede Lecture] the only solution is more knowledge and more wisdom to use this knowledge. There is no limit to the amount of knowledge to be gained if the medical scientist is given the opportunities and facilities for his work. Would that the same could be said about the wisdom necessary to make the best use of this knowledge.

Mellanby was fortunate from the early days of his appointment with the Medical Research Council.

At the time of Mellanby's appointment [in 1933]

says Lord Hankey,

I was Clerk of the Privy Council, to which the Medical Research Council was attached for administrative purposes. I was also Secretary to the Cabinet and to the Committee of Imperial Defense.

In the latter capacity, soon after his arrival, I asked him whether something could be done to improve the health of volunteer recruits for the army, some 50 to 60 per cent of whom were being rejected every year for medical and especially dental reasons. Mellanby, as he recalled to me as recently as January 20th last [1955] replied that the problem could be solved, but only on a long term basis, and by drastic reforms in the national diet. I asked him for a Memorandum on the subject, and to his astonishment, as he also recalled on January 20th, a day or two later it came back to him in the form of a Memorandum to the Cabinet. At the moment, he had been

feeling baffled and exasperated, like many another distinguished Scientist has been, by the well-nigh insuperable difficulty in getting the results of research work translated into action. In terms of rare emotion, at this, our last meeting on earth, he told me that this episode had given him new hope and inspiration.

This episode,

continues Lord Hankey, was

the beginning of our long and valued friendship. It was also a prelude to even closer collaboration, in which Mellanby took a leading part, between the Medical Research Council and other Government Departments concerned, including the Committee of Imperial Defense [when appropriate], with a view to the common aim of safe-guarding our people, should need arise, against those hidden foes of mankind, which in past wars so often brought nations and armies to disaster—disease, malnutrition, contaminated water, biological and microbiological pests of all kinds—the risks of which had been greatly increased by the menace of air bombardment. In the five years before the war, and in the six war years, I was a privileged witness and sometimes a collaborator, as Chairman of Committees or otherwise, in much of that work.[8]

It is quite impossible to do justice to the work which Mellanby accomplished as Secretary of the Medical Research Council. Some idea of the immense scope and intensity of activity may be obtained from reading the Reports of the Medical Research Council for the years 1939 to 1945 and 1945 to 1948. The Council itself, commenting on his period of office, states[13] that it

. . . had been one of exceptional difficulty and strain, during which the Council's programme had to be reorientated to the needs of war and again to those of peace, and the Council's organisation and resources were increased several fold.

The Council recognised the debt which not only they but medical research in general owe to him. They say

He showed a firm grasp of the general principles governing the progress of scientific research. To these he held fast among the multitudinous problems with which he was confronted, so that his advice was far-sighted and his action effective. During his time as Secretary many important medical discoveries have been made or have come to fruition. Modern chemo-therapy and the anti-biotics have revolutionised medical treatment. The science of nutrition has become a major branch of preventive medicine. Personnel research is achieving the recognition its importance deserves. Nuclear physics has brought its profound influence on society. Mellanby's ready appreciation of the significance and possibilities of different lines of research, even when the investigations were in the embryo stage, has been a formative factor in the progress of medical research in this country, and indeed in the world.

In his work, generally, Mellanby was never content unless he could ensure that the practical fruits of research should be reaped. This attitude is found in his work in the international sphere, and from 1930 he was intimately connected with the Health Organization of the League of Nations, and he was Chairman of the international conferences for the standardisation of vitamins in 1931 and 1934 and of the International Technical Commission

[13] Report of the Medical Research Council for the Years 1948–50 (His Majesty's Stationery Office, London, England, 1951).

on Nutrition sponsored by the League. With Professor E. V. McCollum, Mellanby represented nutritional experts on the medical committee of the League of Nations which was responsible for the classical report that led to "the marriage of nutrition with agriculture." The United Nations Organizations now concerned with nutrition owe much to this early spade work, a debt which has probably not been sufficiently acknowledged.

Research abroad owes much to his influence, and in recent years he visited Africa, India, Australia, and New Zealand for the express purpose of advising on research. Earlier he had been to Canada and on other occasions to the United States.

For many years he was active in the development of medical research in British Colonial territories. Not only did he recognise the great need for the application of medical science, but he also foresaw what has only recently been widely recognised as a major problem, namely the need for relating the rapidly increasing population to the supply of sufficient food. He insisted that "... most of the political, social and economic difficulties in tropical countries are and will continue to be of a biological nature and the sooner the fact is recognised, the sooner will these difficulties be controlled or dispersed, and we can only pray that there is sufficient wisdom left among us to use the fruits of science properly" (79).

Philosophy and Motivation

Much of what is written above might well have been included in this section. Inadequate and incomplete as the account may be, there is space for only a few more thoughts. One friend, recalling a lovely moorland walk with "E. M.," remembers the latter saying "There are few subjects you cannot get something out of if you really get down to it." This he followed with a quotation from the last words of the celebrated Laplace "What we know of things is little—what we are ignorant of is immense." "A sobering belief in no finality to knowledge" his friend says, "produced in Mellanby an enthusiasm for work which in turn he was keen to impart to his workers, this making him an ideal leader in research." Glimpses of this attitude are to be found throughout his work; for example, in 1933 in his Cameron Prize Lecture (53) he said "the whole subject of nutrition in relation to tissue structure and function is at present almost uncharted territory." Eleven years later (86) considering biological problems, he says

> Surveying now the general field of nutritional science, what do we find? We see that physiologists and biochemists, mainly by means of experiments on young rats, have detected a large and increasing number of chemical substances which must be present in the diet, often in minute quantities, either for the maintenance of life itself, or for proper growth. The chemical constitution of most of these vitamins is known. The chemists' success has indeed been great. But what about the biological side; can it be said why these substances are essential and/or in what way they work? From this angle the success has not been so outstanding: a biological problem is usually much more difficult than a chemical problem. The living cell in the animal body will not limit itself to actions by the known rules and regulations of the chemist's laboratory. . . . Thus there is hardly any knowledge of how the vitamins work in

performing their function . . . clearly this is a great challenge to all interested in experimental biology—physiologists, biochemists, pathologists, pharmacologists and clinicians.

He recognised to the full "the enormous difficulties of the experimental method in biological investigations due to the complexity of the interacting materials" [Paget Memorial Lecture (78)]. It is of special interest to some of us that in his Hopkins Memorial Lecture he gave an illustration of the difficulties encountered in biological work. He relates that the growth curves shown in Hopkins' publication in 1912 on the nutritive value of tryptophan were reproduced in all parts of the world, yet

. . . neither Hopkins himself nor anybody else was able to repeat the particular experiments—that is, the growth stimulus supplied by 2–3 c.c. of milk per rat per day. There was apparently some unknown condition in the experiments essential for their successful repetition (a state of affairs not uncommon in biological research). This fact . . . greatly troubled Hopkins for many years and it was only in 1945 [over 30 years later] that he returned to the subject and claimed to have found out the cause of the difficulty of repeating the early work. The secret apparently was that in the earlier work he used potato starch in the synthetic diet and this had led to a condition later known as refection (91).

It is perhaps appropriate here to recall his insistence on the proper appreciation of the experimental method: "Most of us know what we mean," he writes, "when we use [the words, observation and experiment] but there is still evidence of confusion in the minds of some people as to the difference between" them. . . . "An observation leads to knowledge of itself, and it is true that, for an experiment to be fruitful, it must be followed by observation. The experiment, however, consists essentially of changing a condition or conditions by active interference whereby any effect produced by these changes can be tested. . . . The words 'good' or 'bad' in connection with an experiment have no meaning at all, except in so far as they reflect the goodness and the badness of the experimenter." "Long experience has taught me," he says in his Harveian Oration, "that it is seldom in the facts of discovery but rather in the interpretation, especially in the first interpretation, mistakes are made (72)." He follows up this theme in a lecture he gave on Jenner in 1949 (93), and his observations on unjust criticism are well worth reading. Amongst his observations is this piece of advice:

If a man thinks that the facts described by an investigator are wrong, then it is no good simply writing about them. The critics must go into the hospital or laboratory, make observations or better experiments in order to prove his contention.

He had one motto he offered to laboratory workers—a positive version of the saying that "chance favours the skilled observer"—it was his injunction to "treasure your exceptions."

He was a fine and indeed a great man and in the words of a contemporary

His greatest titles to fame, however, will still be found in the record of his own major enterprises in research, and of his service to medical research in general, as a great administrator and public official. Other instances could, no doubt, be cited,

beginning perhaps with that of Isaac Newton, of men who, having risen to great eminence as scientific investigators and discoverers, have later found opportunity to show a different aspect of their powers, as great public servants. It was Mellanby's special title to greatness that, having achieved high rank as an investigator of great originality and distinction, he continued to hold it, by maintaining the high level of his own activity in research, when he became, in addition, a great administrator of the public funds provided for the general support of research in his own field of the medical sciences, and a most determined and forceful advocate, in official circles and widely beyond them, of the proper use and application of the results of such research, for the promotion of health in the nation and throughout the world.[1]

With all, to use a sentence written by an old student, "He was so very human," and this is the note on which I would wish to end. He could be pleased and proud as when he found in the distortion of bone growth the explanation of nervous symptoms in his experimental animals, or as when, with enthusiasm and pride, he found, having almost achieved his "three score years and ten" that he could still make discoveries; notably that in high vitamin A plasma, epithelial cells were converted into mucous secreting cells. He could, however, also be pleased, as on the occasion of his election to an Honorary Fellowship of the Royal College of Surgeons of Edinburgh, after several eminent people "had said a few words," when his turn came he said "As a duly qualified surgeon, I now propose to perform here and now my own operation: 'cackle-ectomy'; I shall cut out the cackle." Upon which he sat down.

One quality which I had not, I confess, fully appreciated until re-reading much of his work, was his doggedness. Genius is admittedly rare and if it is no more than an infinite capacity for taking pains, he was indeed a genius. It is some consolation to some of us that he maintained that he was a slow thinker. Those who associated him with quick repartee or a challenging evocative question would tend to be puzzled by his own assessment of himself, but by reading his writings and by appreciating the amount of thought that led to the foreshadowing of some of his discoveries and his general observations, the truth of his own assessment becomes apparent.

Like vitamin A in the growth and modelling of bone he was the "wise director" in the growth and development of medical research and, as will no doubt be found of vitamin A, he was, as can be seen from his personal record of research, an integral part of the operation.

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NONOXIDATIVE AND NONPROTEOLYTIC ENZYMES (AMINO ACID TRANSAMINASES AND RACEMASES)[1]

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A comprehensive review of a wide variety of enzymes of the "nonoxidative and nonproteolytic" group was published in the preceding volume of the *Annual Review of Biochemistry*. The present article is concerned with two somewhat related groups of enzymes which fall within this general category. The increased interest and the rapid progress made in the study of transaminases in the last few years makes consideration of this subject desirable at this time. This review will cover primarily developments reported during the last year or so; the reader is referred to previous reviews for coverage of earlier literature (1, 2, 3). The amino acid racemases are a relatively young group of enzymes, the first of these having been reported in 1951. The subsequent discovery of other amino acid racemases and the finding of a rather impressive number of amino acids of the D-series in nature has opened a new area of biochemical research.

Transaminases

The major recent advances in this field have been (*a*) the recognition that a very large number of amino acids may participate in transamination reactions, (*b*) the development of further information concerning the mechanism of transamination, and (*c*) the acquisition of evidence relating to the role of transamination in amino acid metabolism. There is room for considerable progress in the area of enzyme purification. The vitamin B_6-Schiff base mechanism, although supported by much evidence and consistent with many observations, has yet to be proven conclusively.

Scope of the transamination reaction.—Further evidence emphasizing the wide variety of susceptible substrates has continued to accumulate. Roberts has reported that extracts of animal tissues and bacteria were capable of forming glutamic acid from α-ketoglutaric acid and δ-aminovaleric acid or α,γ-diaminoglutaric acid (4). As yet, the latter amino acid is not known to occur naturally. On the other hand, δ-aminovaleric acid is known to be a decarboxylation product of α-keto-ϵ-aminocaproic acid (5), and there is evidence consistent with the formation of the latter compound from lysine in animal and plant tissues (6, 7). Suda *et al.* (8) have presented evidence for transamination between δ-aminovaleric acid and α-ketoglutaric acid in preparations of lysine-adapted soil bacteria. The formation of glutaric semialdehyde was not demonstrated, although it was postulated that the semialdehyde was formed and underwent further degradation via glutaric

[1] The survey of the literature pertaining to this review was concluded in October, 1955.

acid by the intact cells. Such a reaction sequence is similar to that shown to take place in guinea pig liver by the isotope studies of Borsook *et al.* (9). The transamination reaction of δ-aminovaleric acid is analogous to that of γ-aminobutyric acid and α-ketoglutaric acid, which yields succinic semialdehyde and glutamic acid (10). Malonic semialdehyde formation in the β-alanine-α-ketoglutaric acid reaction (10, 11) has not yet been demonstrated.

Scher & Vogel (12) have studied the ornithine-α-ketoglutaric acid reaction in a wide variety of microorganisms. *Neurospora sitophila*, *Torula utilis*, *Bacillus subtilis*, baker's yeast, and *Bacillus pumilus* were found to be capable of catalyzing this reaction, which yields glutamic-γ-semialdehyde and glutamic acid, while *Escherichia coli*, *Aerobacter aerogenes*, *Proteus vulgaris*, and several others were inactive. Vogel (13) and Davis (14) have discussed the biosynthetic significance of this reaction.

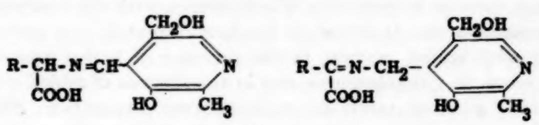
The demonstration of the participation of D-amino acids in transamination in certain bacteria by Thorne and collaborators (15 to 18) represents a highly interesting finding which helps to explain the formation of D-glutamyl polypeptides in *B. subtilis* and related organisms. Although participation of D-amino acids in transamination was reported in rather early studies, these studies usually suffered from the lack of highly purified amino acid isomers and good methods for amino acid isomer detection. Thorne found that cell-free extracts of *B. subtilis* catalyzed the formation of D-glutamic acid from α-ketoglutaric acid and D-alanine or D-aspartic acid. Fresh extracts were active with both D- and L-aspartic acid, and when the L-isomer was used, the product was predominantly L-glutamic acid. After ageing and dialysis of the extracts, the D-transaminase activity was much greater than the L activity, and a requirement for pyridoxal phosphate could be demonstrated. Fractionation of the extract with ammonium sulfate led to a preparation which was active only with D-isomers. The evidence, therefore, supports the existence of a D-specific transaminase, rather than one with no or low optical specificity. Further work has shown that the D-transaminase can catalyze the reaction in both directions and that other D-amino acids are also active in transamination. Thus, D-methionine and D-serine were active in forming D-glutamic acid with the *B. subtilis* enzyme. Extension of these studies to *B. anthracis* (18) has led to the finding that D-phenylalanine, D-tryptophan, D-methionine, D-histidine, and D-leucine are capable of transaminating with pyruvic acid to yield D-alanine. It is curious that, in general, α-ketoglutaric acid did not serve in place of pyruvic acid in this system. The most active reaction observed was between pyruvic acid and D-phenylalanine. The only D-isomer which reacted with α-ketoglutaric acid was D-alanine, while the only L-isomer which was active with pyruvic acid was L-glutamic acid. Thorne and collaborators believe that the D-configuration is produced originally by alanine racemase (see below, p. 46) which is also present in the organisms which possess D-transaminase activity. It would obviously be of great interest to know in what way the D-trans-

aminase molecule differs from the L-specific-enzyme. The possibility that the D-specific enzyme contains D-amino acid residues may be susceptible to investigation, provided that the enzyme is obtained in a high state of purity.

The investigation of transamination of D-amino acids has been impeded until relatively recently by the unavailability of pure D-isomers. Unfortunately many investigators have used racemic amino acids in studies on transamination and assumed, without adequate proof, that only the L-enantiomorphs were active. Early reports of the participation of D-amino acids in transamination (1, 2) were criticized, probably justly, on the basis that the isomers used contained significant quantities of the respective enantiomorphs. Recently, Cedrangolo (19), in referring to earlier work from his laboratory (20), has stated that optically pure D-amino acids were used in studies which purported to show that animal tissues, including brain and lung, were capable of catalyzing D-transamination. It would be of importance to repeat these experiments using enzymatic determinations of optical purity of the amino acids used.

Studies on the mechanism of transamination.—There is considerable evidence for the participation of vitamin B_6 in transamination, although neither of the presumed coenzymes, pyridoxal phosphate and pyridoxamine phosphate, has been isolated from a transaminase and characterized. The observation that pyridoxal phosphate and pyridoxamine phosphate are equally active in restoring the enzymatic activity of a resolved transaminase (21) is consistent with the Schiff base mechanism originally suggested by Snell (22). Indirect support for this hypothesis has come from studies on nonenzymatic transamination between amino and α-keto acids and the aldehyde and amine forms of vitamin B_6. The necessity for the presence of a metal ion for nonenzymatic transamination was demonstrated by Snell and collaborators (cf. 23). The question as to the participation of a metal ion in the enzymatic reaction must probably await studies on purified transaminases.

Eichhorn & Dawes (24) have carried out a very interesting study of the nonenzymatic reaction between pyridoxal and alanine in the presence of metal ions. By following the absorption spectrum of reaction mixtures over a period of time, these workers obtained evidence for the formation of the metal complexes of two interconvertible Schiff bases:



They demonstrated that the end product is the same when copper (or nickel) ions are mixed with either pyridoxal and alanine or pyridoxamine and pyruvic acid. However, the initial spectrum of the pyridoxamine-pyruvate-metal ion mixture was different than that observed with the pyridoxal-

alanine-metal ion mixture. The spectrum of the former reaction mixture gradually shifted to that of the latter, indicating that, under the conditions employed, the equilibrium is in favor of transamination from pyridoxamine to pyruvic acid.

Using a paper electrophoretic technique, Fasella *et al.* (25) have obtained evidence for the Schiff base intermediates in nonenzymatic transamination between pyruvic acid and pyridoxamine and between alanine and pyridoxal. Two Schiff base chelates, CI and CII, were located by means of their strong fluorescence, and further characterized by their absorption spectrum and chromatographic and electrophoretic behavior. On hydrolysis, CI gave pyridoxal and alanine, while CII yielded pyridoxamine and pyruvic acid. Neither exhibited properties characteristic of a free amino or aldehyde group, and it was further shown that CII could be converted to CI. These studies, and those of Eichhorn & Dawes (24), provide excellent support for the Schiff base mechanism in nonenzymatic transamination.

Nonenzymatic reactions of ornithine and lysine have been studied by Witkop & Beiler (26), who reported that the ω-amino groups of these amino acids readily condense with aromatic aldehydes. It is of interest that attempts to condense ω-carbobenzoxylysine with benzaldehyde or salicylaldehyde were not successful. The only enzymatic transamination reactions of ornithine which are now known involve the δ-amino group (27 to 30), although transamination involving the α-amino group has been demonstrated when the ω-amino group of ornithine or lysine is substituted (28, 30).

Nonenzymatic transamination between peptides and pyridoxal has been reported by Cennama (31). Leucylglycine, leucylglycylglycine, alanylglycine, and alanylalanine were found to react with pyridoxal in the presence of aluminum ions at 100°C. to yield pyridoxamine; formation of the corresponding keto-peptides was not reported. Nonenzymatic transamination in the reverse direction, i.e., pyruvoylalanine to alanylalanine, was reported a number of years ago by Herbst & Shemin (32). The question as to whether analogous enzymatic reactions occur is still an open one (see p. 43).

Hilton *et al.* (33) have contributed further to their interesting series of studies on the mechanism of enzymatic transamination. They have attempted to define the relationship of the exchange of the α-hydrogen atom of the amino acid to the process of transamination. Earlier studies (1, 2, 3) showed that there is an exchange of α-hydrogen with the aqueous medium during transamination. However, it has been reported that such exchange may occur with boiled enzyme, in the presence of amino and keto acids which are not active as substrates, and in the absence of added α-keto acid. Using a crude α-ketoglutarate-aspartate enzyme preparation, Hilton *et al.* (33) found that the glutamate enzymatically formed in the presence of D_2O contained approximately one molecule of deuterium. Aspartate hydrogen was also replaced during the reaction; however, less than 6 per cent as much aspartate hydrogen exchanged when α-ketoglutarate was omitted. This result suggests that the rapid hydrogen exchange of aspartate and glutamate

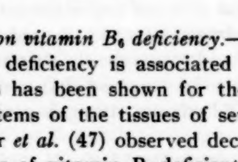
are closely associated with the action of the enzyme; and indeed such a result is consistent with the concept that there are common sites on the enzyme for either pair of reactants. In a similar series of experiments by Peyser (34), in which a purified and resolved glutamate-aspartate enzyme was used, the incorporation of deuterium into glutamate was also close to 1 mole; the results were the same with either pyridoxal phosphate or pyridoxamine phosphate. Peyser (34) and also Nisonoff *et al.* (35) have observed enzymatic transamination between an amino acid and its own α-keto analogue. This been shown with alanine and C^{14}-pyruvate and with C^{13}-glutamate and α-ketoglutarate (34, 35). These findings indicate that in a system containing two keto acids and their amino acid analogues, transaminations occur between compounds possessing identical carbon chains, a phenomenon which would be expected to decrease the rate of the reaction as determined by new amino or keto acid formation. Evidence for such a retardation in rate of transamination was observed by Nisonoff *et al.* (36) in earlier studies.

The exchange of the α-deuterium atom of amino acids during enzymatic transamination has been utilized for the preparation of α-deuterium-labeled amino acids (37, 38).[2] Grisolia & Burris (38) have now synthesized α-ketoglutarate labeled with deuterium in the β-position. Enzymatic transamination of β-deutero-α-ketoglutarate with cysteine sulfinic acid at pH 9.4 led to glutamate containing about 26 per cent less deuterium. On the other hand, in experiments with the α-ketoglutarate-alanine system at pH 7.2, the glutamate formed contained approximately the same concentration of deuterium as the α-ketoglutarate. A greater loss of deuterium from α-ketoglutarate would be expected at more alkaline pH because of an increased tendency to enolization (cf. 42). The observation that the β-hydrogen atoms of glutamate and α-ketoglutarate do not exchange during transamination is consistent with earlier work on deuterium-labeled leucine and on enzymatic transamination of L-isoleucine and L-alloisoleucine. Sprinson & Rittenberg (41) fed rats leucine labeled with deuterium in α-, β-, and γ-positions; the β- and γ-deuterium atoms of the leucine isolated from the tissues of these animals were diluted to approximately the same extent. Studies on transamination of L-alloisoleucine and L-isoleucine in animal and bacterial systems yielded the pure *l*- and *d*-isomers, respectively, of α-keto-β-methylvaleric acid (30, 42). Thus there are now several pieces of experimental evidence in support of the concept that the β-hydrogen atom of the substrate is not rendered labile during transamination, and consequently that an α, β-dehydrogenation does not occur. It should also be observed that α-aminophenylacetic acid, which does not possess a β-hydrogen atom, is active in transamination with α-ketoglutarate (30).

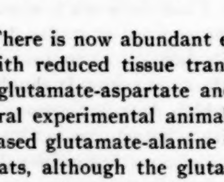
The ability of ω-methylpyridoxal, ω-methylpyridoxamine, and ω-methyl-

[2] Transamination has not been widely utilized for preparative purposes, although nonenzymatic transamination has been employed for the preparation of α-ketoisocaproic acid (39). Pfleiderer (40) has used coupled glutamate-alanine and glutamic dehydrogenase systems for the determination of alanine and glutamate.

pyridoxamine phosphate to replace vitamin B_6 in supporting the growth of *Streptococcus faecalis* under growth conditions necessitating synthesis of leucine, isoleucine, phenylalanine, valine, tyrosine, methionine, or serine, suggested that ω-methylpyridoxal phosphate and ω-methylpyridoxamine phosphate might serve as active coenzymes for transaminase (43). It was subsequently found that ω-methylpyridoxal phosphate was capable of activating the leucine-glutamate, phenylalanine-glutamate, tyrosine-glutamate, and alanine-glutamate activities of a cell-free preparation of *S. faecalis;* however the apoenzymes usually exhibited a lower affinity for ω-methylpyridoxal phosphate than for pyridoxal phosphate. The ω-methyl analogue did not produce appreciable activation of the cysteine desulfhydrase activity of such preparations. It is of some interest that the methyl group of pyridoxal may be replaced by an ethyl group without complete loss of biological activity. It is also noteworthy that the relative affinities of different vitamin B_6 enzyme systems for pyridoxal and ω-methylpyridoxal phosphates vary considerably. Thus, the ratio of the activities of pyridoxal phosphate to ω-methylpyridoxal phosphate for the alanine racemase and leucine-glutamate transaminase systems was 10, while for phenylalanine- and tyrosine-glutamate transaminase activities it was about unity. It would be of interest to know whether apoenzymes reconstituted with ω-methylpyridoxal are more readily resolved than those reconstituted with vitamin B_6 coenzyme. The purified glutamate-aspartate apoenzyme of pig heart could be freed from 4-deoxypyridoxine phosphate and pyridoxamine phosphate by dialysis, while loss of pyridoxal and pyridoxamine phosphates was not observed under these conditions (21).



Pyridoxal phosphate



ω-Methylpyridoxal phosphate

Studies on vitamin B_6 deficiency.—There is now abundant evidence that vitamin B_6 deficiency is associated with reduced tissue transaminase activity. This has been shown for the glutamate-aspartate and glutamate-alanine systems of the tissues of several experimental animals (3, 44, 45, 46). Meister *et al.* (47) observed decreased glutamate-alanine transaminase in the livers of vitamin B_6-deficient rats, although the glutamine-phenylpyruvate and glutamine-pyruvate transaminase-deamidase activities of these livers was not significantly different from those of the control (non-deficient) animals. This finding could be interpreted to mean that vitamin B_6 is not involved in the glutamine-α-keto acid system, or alternatively, that the affinity of the glutamine system for vitamin B_6 is greater than that of the

glutamate-alanine system. More recent studies (48) support the latter interpretation. It was found that administration of isonicotinic acid hydrazide to rats led to a marked reduction in the activities of both the liver glutamate-alanine and glutamine-α-keto acid transaminase systems. Furthermore, on addition *in vitro* of either pyridoxal phosphate or pyridoxamine phosphate, both activities were restored to levels approximately the same as those of the controls. The administration of isonicotinic acid hydrazide apparently causes, in addition to other effects, a depletion of vitamin B_6. Presumably, the hydrazide acts by combining with the aldehyde group of pyridoxal (cf. 49, 50). There is evidence for the urinary excretion of vitamin B_6, probably as the hydrazone, after the administration of isonicotinic acid hydrazide to humans (51). It is probable that this compound has a number of other effects, especially when given in large doses, and it is therefore conceivable that lowered transaminase activity might be attributable, at least in part, to an effect of the drug on apoenzyme formation. The observation that pyridoxal and pyridoxamine phosphates have an *in vitro* activating effect is therefore particularly significant. The livers of rats or mice given a vitamin B_6-deficient diet for very long periods exhibit decreased glutamine and glutamate transmainase; however, *in vitro* addition of pyridoxal phosphate does not produce activation (48).

It thus appears that at least one mechanism for the deamidation of glutamine may depend upon the presence of vitamin B_6. It is probably the first step of the transamination-deamidation reaction, i.e., transamination of glutamine to α-ketoglutaramic acid, which is vitamin B_6-dependent. The second step, in which the ammonia is released, is hydrolysis of α-ketoglutaramate to α-ketoglutarate (52). Beaton & Goodwin (53) have reported that vitamin B_6-deficient rats exhibited a lowered renal phosphate-activated glutaminase. They also report restoration of activity by *in vitro* addition of pyridoxal phosphate. This could be interpreted to mean that glutaminase may be a vitamin B_6 enzyme; however, the difference between the mean glutaminase activities of control (35 ± 4) and deficient (27 ± 7) groups was not great, nor was the effect of pyridoxal phosphate (with pyridoxal phosphate, 38 ± 7; without pyridoxal phosphate 27 ± 7) as striking as in comparable experiments with transaminase. Beaton & Ozawa (54) have examined the liver phosphate-activated glutaminase activity of vitamin B_6-deficient rats. In contrast to results with kidney, deficiency was associated with a moderately increased glutaminase (42.9 ± 5.3 compared to 31.6 ± 6.8 of the controls); *in vitro* addition of pyridoxal phosphate reduced the activity of both deficient (32.2 ± 9.5) and control (24.0 ± 10.1) tissues, although the effect on the controls was not considered significant. Beaton & Ozawa also observed a decrease in the rate of deamidation of glutamine in the presence of pyruvate by the livers of rats deficient in vitamin B_6; addition of pyridoxal phosphate to the homogenates produced small and inconstant activation (54). Other evidence (48) suggests that the glutamine-α-keto acid reaction, like other transamination reactions, requires vitamin B_6 as a coenzyme. Although the relationship of vitamin B_6 to the renal and hepatic phosphate-

activated glutaminases appears uncertain because of the relatively small effects observed in crude homogenate systems, vitamin B_6 deficiency may affect glutamine deamidation indirectly through the glutamine-α-keto acid transaminase system. Whether the effect of vitamin B_6 deficiency on this system is responsible for the decreased plasma glutamine level (55) and increased liver and kidney glutamine levels (54) of vitamin B_6 deficient-rats is not clear. Vitamin B_6 functions as a coenzyme in a great many reactions, and therefore deficiency of this vitamin should be associated with a multitude of effects.

It is also evident that transaminases may exhibit wide variation in their affinity for coenzyme. This is clear from the studies on glutamine-keto acid transamination discussed above (47) and also from recent work on the transamination of cysteine sulfinic acid. Chatagner *et al.* (56) found that liver preparations obtained from vitamin B_6 deficient-rats exhibited much lower cysteine sulfinite-pyruvate transaminase activity than that of controls, whereas the deficiency did not affect the levels of cysteine sulfinate-α-ketoglutarate and glutamate-pyruvate transaminase activities.

Metabolic significance of transamination.—There is now considerable information on the ability of the α-keto acid analogues of certain amino acids to support growth of animals and microorganisms in place of the corresponding amino acids (cf. 3). It is probable that transamination is a major reaction involved in the synthesis of amino acids in these studies. Recently, Wood & Cooley (57) demonstrated that young rats grew as rapidly on a diet consisting of 10 essential amino acids plus glutamic acid, as when fed a ration in which leucine, isoleucine, valine, phenylalanine, and methionine were all replaced by their α-keto acid analogues and an equivalent nitrogen source consisting of glycine and aspartic acid. This suggests that the potential transaminase activity of the intact rat may be considerable; in view of studies demonstrating the necessity for the simultaneous presence of all of the amino acids for protein synthesis, it may be concluded that the conversion of these five α-keto acids to the analogous amino acids takes place rapidly.

Although it has become increasingly apparent that transamination reactions are of considerable metabolic importance, attempts to relate transamination directly to such phenomena as protein synthesis, growth, and development have not been successful. On the other hand, the role of transamination in the biosynthesis and metabolic degradation of certain amino acids has been established. That transamination might play a role in the oxidative deamination of amino acids was first suggested by Braunstein & Bychkov in 1939 (58). The suggested mechanism (see also 1, 59) involved a coupled reaction between a α-ketoglutarate-amino acid transaminase and the glutamic dehydrogenase system. Since these reactions are reversible it would be expected that amination of α-keto acids might also take place by this mechanism:

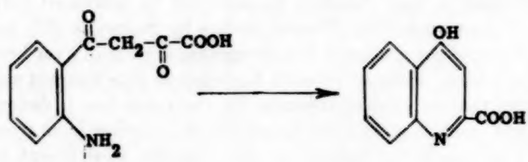
$$\alpha\text{-ketoglutarate} + \text{L-amino acid} \rightleftharpoons \alpha\text{-keto acid} + \text{L-glutamate}$$
$$\text{L-glutamate} + \tfrac{1}{2}O_2 \rightleftharpoons \alpha\text{-ketoglutarate} + NH_3$$
$$\text{Sum: L-Amino Acid} + \tfrac{1}{2}O_2 \rightleftharpoons \alpha\text{-Keto Acid} + NH_3$$

According to this scheme, the only enzyme system to catalyze direct amination or deamination of an L-amino acid is the glutamic dehydrogenase system. The L-amino acid oxidase system of rat kidney is relatively low in activity, and its physiological significance has therefore been questioned (60). However, the question as to whether reversible amination systems exist for amino acids other than L-glutamic acid deserves study, and the existence of such systems cannot yet be excluded (cf. 61). Specific enzyme systems, such as those catalyzing the deamination of threonine, serine, and cysteine have been reported. In an interesting discussion of this problem, Cedrangolo (19) has concluded that although deamination of amino acids may take place by the coupled mechanism outlined above, there is evidence for the existence of an enzyme system that directly oxidizes one or more of the natural amino acids. Cedrangolo observed that liver and kidney pulps catalyzed the oxidation of L-alanine (as observed by measurement of oxygen consumption and ammonia formation) more rapidly than L-glutamic acid. In other studies, it was found that addition of α-ketoglutarate to liver preparations (rat, rabbit, pigeon, hen) did not increase the deamination of L-alanine, and in some cases the formation of ammonia was reduced in the presence of α-ketoglutarate. However, it is evident that these results do not necessarily support the existence of an alanine deaminase; suppression of ammonia formation may possibly be ascribed to increased formation of glutamate by transamination. Recent studies by Sinitsyna (62) have shown that the activity of the glutamic dehydrogenase system in liver homogenates and liver and kidney slices of vitamin B_6-deficient rats was not significantly different from that of control animals. On the other hand, deamination of L-alanine and L-aspartate, and the formation of L-alanine from pyruvate and ammonia, were markedly reduced in the vitamin B_6-deficient tissues. In confirmation of many earlier studies, it was observed that several transamination reactions also proceeded at very low rates, and Sinitsyna has concluded that the amination of α-keto acids other than α-ketoglutarate occurs principally by transamination. There appears to be little doubt that coupled glutamic dehydrogenase-transaminase reactions can occur. However, it seems possible that other mechanisms may exist for the deamination and re-amination of amino acids, at least in certain cells (cf. 63, 64, 65). It is well known that certain plants can incorporate ammonia into aspartate by the aspartase reaction. Marr *et al.* (66) have made an interesting study of the oxidation of glutamate by *Brucella abortus*. This organism converts glutamate to pyruvate, alanine, carbon dioxide, and ammonia. When the oxidation was permitted to take place in the presence of N^{15}-ammonia, the alanine formed was found to contain a higher concentration of isotope than the glutamate; the concentration of N^{15} in the alanine was, however, lower than that of the ammonia added originally. From these studies, the authors conclude that about 75 per cent of the alanine may be formed by transamination of pyruvate with glutamate and that the remainder of the alanine arises by other mechanisms. It is possible, though not proven by these experiments, that some alanine may arise by direct amination of pyruvate.

Although there has been much discussion concerning direct amination of α-keto acids and of the oxidative deamination of L-amino acids, and some experimentation, (usually with systems which also exhibit transaminase acitivity), it seems probable that studies with purified tissues fractions will be important for the final solution of the problem. Thus far, the only general deamination system which has been studied in animal tissues is the L-amino acid oxidase of Blanchard *et al.* (67).

In contrast to the problem of the mechanism of oxidative deamination of amino acids, there is less uncertainty concerning the role of transamination in the metabolism of certain individual amino acids. Only some of the more recent work will be considered here; earlier studies have been discussed elsewhere (3).

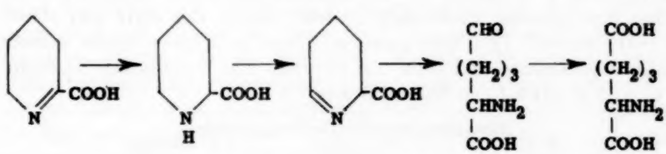
The participation of transamination in the conversion of kynurenine to kynurenic acid now appears well established. In studies on hog liver kynureninase, Wiss (68) observed that kynurenine was converted to kynurenic acid provided that either pyruvic acid or α-ketoglutaric acid was present (see also 69). Miller *et al.* (70) subsequently found that a strain of *Pseudomonas* was capable of catalyzing transamination between kynurenine and α-ketoglutaric acid to form kynurenic and glutamic acids. Presumably kynurenic acid is formed by spontaneous cyclization of



o-aminobenzoylpyruvic acid, a reaction which is somewhat analogous to the conversion of α-keto-ϵ-aminocaproic acid to Δ^1-piperidine-2-carboxylic acid (5). However, in contrast to the latter reaction, the formation of kynurenic acid is not reversible, because of the formation of a stable aromatic ring. Transamination of kynurenine represents, in animal tissues as well as in certain bacteria, a reaction which competes with kynureninase; both reactions require pyridoxal phosphate as a coenzyme. Mason (71) observed that rat liver kynureninase was relatively weak at pH 6.3, and that at this value of pH considerable transaminase activity could be demonstrated, and Wiss (72) has effected a physical separation of the kynureninase and kynurenine transaminase of rat liver. Rat kidney apparently possesses considerable kynurenine transaminase activity (71). The enzyme appears to be especially unstable, which has thus far prevented its purification; however, the presence of α-ketoglutarate was found to decrease the rate of inactivation to a considerable extent. The presence of kynurenine transaminase activity in kidney is somewhat puzzling since kidney preparations apparently do not effect an appreciable conversion of tryptophan to kynurenine or kynurenic

acid, nor are there significant amounts of kynurenine in the blood under normal conditions (73, 74). The physiological significance of this activity remains to be investigated, as does that of its remarkable instability. The formation of xanthurenic acid from 3-hydroxykynurenine also takes place by transamination; the reaction is analogous to the transamination of kynurenine (68, 75). Kynurenine transaminase preparations also act on 5-hydroxykynurenine; Hayaishi (75) has reported that transamination between 5-hydroxykynurenine and α-ketoglutaric acid yields a compound considered to be 6-hydroxykynurenic acid on the basis of ultraviolet absorption studies (see also 75a).

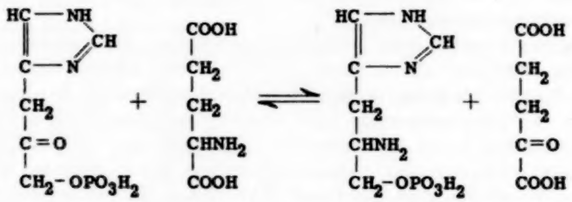
There is as yet no definitive enzymatic work on the transamination of lysine. As mentioned above, there is evidence that α-keto-ϵ-amino-caproic acid (or its cyclized form, Δ^1-piperidine-2-carboxylic acid) is formed from lysine (5, 6). Such a conversion could occur by transamination or by oxidative deamination. Clark & Rittenberg (76) found that labilization of the α-deuterium atom of lysine in the intact rat was slight compared with that of leucine or glycine and concluded that "lysine is not involved in the transaminase system." On the other hand, these studies do not exclude nonreversible transamination of lysine to its keto analogue. In the rat, Rothstein & Miller (77) have found that pipecolic acid is a major metabolic product of lysine and the conversion of lysine to pipecolic acid involves loss of the α-amino group of lysine, rather than the ϵ-amino group. They have also suggested that the available evidence is consistent with an intramolecular transamination reaction in which the ϵ-amino group of lysine be-becomes the α-amino group of α-aminoadipic acid. By this mechanism Δ^1-piperidine-2-carboxylic acid would yield pipecolic acid and Δ^1-piperidine-6-carboxylic acid, which could open to α-aminoadipic acid-δ-semialdehyde:



Studies on the individual reactions involved have not yet been made. The conversion of lysine to α-keto-ϵ-aminocaproic acid can occur by oxidative deamination, and it is of interest that this reaction takes place more rapidly when the ϵ-amino is substituted. The observation that ϵ-N-substituted lysine can support the growth of rats in place of lysine (78) is consistent with the suggestion that a similar derivative is involved in its metabolism. Transamination of ϵ-N-substituted lysine also takes place more rapidly than that of lysine itself (28, 30). Although several studies have suggested that lysine can participate in enzymatic transamination *in vitro* (79, 80), the fate of the lysine carbon chain has not been determined.

Ames has continued his study of the unique transamination reaction involved in histidine synthesis in *Neurospora crassa* (81 to 84). In earlier work, evidence was obtained for the following biosynthetic sequence:

Imidazoleglycerol phosphate→Imidazoleacetol phosphate→Histidinol phosphate→histidinol→histidine. Ames made the interesting discovery that the second step could be accomplished by a transamination reaction between imidazoleacetol phosphate and glutamate yielding histidinol phosphate and α-ketoglutarate:



This finding is of significance in the biosynthesis of histidine, and in demonstrating the participation of a phosphate ester in transamination. An analogous reaction is the interconversion between pyridoxal phosphate and pyridoxamine phosphate, which presumably takes place on the enzyme surface. Another similar reaction is transamination of pyridoxamine phosphate with α-keto acids (85). Other molecules such as certain sugar phosphate derivatives, ethanolamine phosphate, etc., may also participate in transamination. Transamination of a compound which possesses both carboxyl and phosphate ester functions, e.g., phosphohydroxypyruvate, might represent an interesting problem (cf. 3). The histidinol phosphate transaminase activity has been purified about eighteen-fold, and in this state was about 90 per cent resolved. Pyridoxal phosphate, and to a lesser extent ω-methylpyridoxal phosphate activated the enzyme. The equilibrium constant at 37° C. and at pH 8.1 (optimal pH) was

$$K = \frac{[\text{Histidinol Phosphate}][\alpha\text{-Ketoglutarate}]}{[\text{Imidazoleacetol Phosphate}][\text{glutamate}]} = 0.04.$$

It was found that neither L-histidinol nor imidazoleacetol was active in place of the corresponding phosphate esters. On the other hand, L-α-aminoadipate, L-arginine, and L-histidine were capable of replacing glutamate, although they exhibited somewhat less than half of the activity observed with glutamate. Similarly, it was observed that α-ketoadipate and α-keto-δ-guanidinovalerate were active in the reverse reaction with histidinol phosphate; pyruvate and oxalacetate were inactive. The enzyme preparation also catalyzed α-aminoadipate-glutamate, glutamate-arginine, glutamate-histidine, α-aminoadipate-arginine, and α-aminoadipate-histidine transamination reactions. It seems probable that all of these relations are catalyzed

by a single enzyme. Furthermore, there was no evidence for the presence of glutamate and α-ketoglutarate in the enzyme preparation, rendering coupled glutamate transamination reactions improbable. The concept that a single enzyme may be capable of catalyzing reactions between any two of several susceptible substrates was suggested by Rudman & Meister (86), who postulated that a single enzyme catalyzed glutamate-isoleucine-leucine-valine transamination reactions in *E. coli*. The possibility of a phenylalanine-tyrosine-tryptophan-glutamate transaminase was also considered. The histidinol phosphate-histidine-arginine-glutamate-α-aminoadipate system of *N. crassa* may represent another such case.

There is now evidence that cysteine itself and two oxidation products of this amino acid (cysteine sulfinic acid and cysteic acid) participate in transamination. The elegant studies of Singer & Kearney (87, 88) have resulted in demonstration of transamination between cysteine sulfinic acid and α-ketoglutarate (or oxalacetate) in bacteria and in animal tissues. The products of the reaction are glutamate (or aspartate) and β-sulfinylpyruvate. The latter compound spontaneously decomposes (in analogy with oxalacetate) to pyruvate and sulfite. These experiments appear to provide an explanation for the previously observed conversion of cysteine sulfinic acid to alanine and sulfite (89); the pyruvate formed in the reaction is converted to alanine by transamination with glutamate (90). Transamination of cysteine sulfinic acid is a very rapid transamination reaction, and it is of particular interest that the purified glutamate-aspartate enzyme preparation of pig heart catalzyes the α-ketoglutarate-cysteine sulfinate reaction more rapidly than the α-ketoglutarate-aspartate reaction. This raises the interesting question as to whether both reactions are catalyzed by a single enzyme. The solution of this problem must probably await further developments in transaminase purification. However, in analogy with the transaminases of *E. coli* (86) and *N. crassa* (84), the enzyme usually designated "glutamate-aspartate transaminase" could justifiably be named "cysteine sulfinate-glutamate-aspartate transaminase." The structural similarity of the substrates and the available enzymatic data support such a designation. It has been previously observed that this enzyme system can catalyze glutamate-mesoxalate, α-ketoglutarate-cysteate, and γ-methylglutamate-oxalacetate transamination reactions (2, 3). It is evident that the glutamate-asparate enzyme can no longer be regarded as highly specific for glutamate and aspartate and their α-keto acid analogues. The question of nomenclature of the transaminases thus becomes a difficult one, especially in cases where a given system may catalyze reactions between a number of substrates.

Singer & Kearney (88) have observed that whereas the cysteine sulfinate-oxalacetate reaction went to completion, the cysteine sulfinate-α-ketoglutarate reaction proceeded only to about 50 per cent of completion, despite removal of the α-keto acid product. Grisolia & Burris (38) were likewise unable to shift the equilibrium of the aspartate-α-ketoglutarate reaction

by decarboxylation of oxalacetate. The explanation of these results is not now apparent.

Although cysteate can react with α-ketoglutarate with the glutamate-aspartate enzyme preparation (2), Darling (91) suggests that there is a separate enzyme responsible for catalysis of this reaction. The conversion of sulfopyruvate to cysteate by transamination with glutamate (91) and with glutamine (92) has also been demonstrated.

Studies on transamination of cysteine have been complicated by the fact that many cell preparations (e.g., pancreas, liver, kidney, and certain bacteria) exhibit cysteine desulfhydrase activity. The products of this reaction, first observed by Fromageot *et al.* (93), include pyruvate, ammonia, and hydrogen sulfide. Kallio (94) has shown that the cysteine desulfhydrase system of *Proteus morganii*, which is activated by pyridoxal phosphate, yields the three products in stoichiometric amounts. The participation of pyridoxal phosphate in the reaction is suggested by experiments where vitamin B_6 deficiency was associated with reduction of enzyme activity; the addition of pyridoxal phosphate to extracts of deficient tissues resulted in restoration of activity. A mechanism of the reaction, based on Schiff base formation between pyridoxal and cysteine has been proposed, and an analogous non-enzymatic model has been devised (23). Although this formulation is consistent with the observations, it should be noted that little success has yet attended attempts to purify cysteine desulfhydrase. Furthermore, some of the available data indicate that formation of equivalent amounts of the products is not always observed. Tamiya (95, 96) has found that the aerobic decomposition of cysteine in *E. coli* occurs in two stages, the first of which may be a deamination of cysteine to β-mercaptopyruvate. Ichihara *et al.* also report a primary liberation of ammonia followed by hydrogen sulfide formation, although these workers do not belive that β-mercaptopyruvate is an intermediate (97). The conversion of β-mercaptopyruvate to pyruvate and sulfur (and, in the presence of reducing substances, to hydrogen sulfide) was demonstrated in several animal tissues and microorganisms [including *E. coli* (92)]. Under conditions whereby conversion of β-mercaptopyruvate to pyruvate did not occur, it was possible to demonstrate transamination of the keto acid to cysteine (or cystine). This has been demonstrated in the glutamine transaminase system of rat liver. The formation of cysteine [demonstrated by the Sullivan reaction (98)] by transamination of glutamate and β-mercaptopyruvate was shown with preparations of *E. coli* (99). Studies with β-mercaptopyruvate are complicated by the occurrence of non-enzymatic reactions which appear to represent condensation of cysteine (and β-mercaptopyruvate) and pyruvate (92, 99; cf. 100); further study of these reactions is necessary. Transamination between cysteine and α-ketoglutarate to yield glutamate has also been reported (92, 101). The available evidence suggests that cysteine can participate in transamination reactions and that β-mercaptopyruvic acid may therefore be an intermediate in cysteine metabolism. This pathway deserves consideration in certain systems

in which desulfuration of cysteine appears to occur in two steps. It does not seem probable that β-mercaptopyruvate is an intermediate in certain "cysteine desulfhydrase" reactions in which ammonia is formed in stoichiometric quantities. However, the cysteine desulfhydrase reaction is a complex reaction, thus far only observed in relatively crude tissue preparations, and it is probable that further study will be needed to elucidate this mechanism as well as to evaluate the possible participation of transamination.

Studies on the transamination of glutamine and asparagine with α-keto acids have led to the conclusion that these reactions occur in two steps, the first of which is transamination to yield the α-keto analogues of the amino acid-ω-amides. The second reaction is enzymatic deamidation of the α-keto acid-ω-amides by an enzyme system which has no activity toward the amino acid-ω-amides (102). These studies have been considered in detail elsewhere (103). In the course of this work it was found that α-ketoglutaramic and α-ketosuccinamic acids could each exist in two interconvertible forms, only one of which exhibited enzymatic susceptibility and properties characteristic of typical α-keto acids. A discussion of the problem of the structures of the unreactive forms of these α-keto acid-ω-amides has been presented (104); proof of structure has yet to be offered. Otani & Meister (104) have reported the preparation of a series of related compounds, e.g., β-oxaloacetylglycine, γ-(α-ketoglutaryl)-alanine. These derivatives may also exist in an unreactive form; however, transamination of β-oxaloacetylglycine and of β-oxaloacetylalanine, which exist predominantly in open chain form, to β-aspartylglycine and β-aspartylalanine was demonstrated in the glutamine transaminase system. These studies extend the scope of known enzymatic transaminations to molecules which possess a peptide bond. It may be observed, however, that transamination of α-peptides, although shown in nonenzymatic systems, has not yet been observed enzymatically. The glutamine transaminase system, which exhibits a relatively narrow specificity for the amino donor (homoglutamine and asparagine are inactive), is nevertheless capable of aminating a very wide variety of α-keto acids. Recently it has been observed that α-keto-γ-hydroxybutyrate is aminated to homoserine in this system (105). The natural occurrence of this keto acid has recently been reported (105a). A dimer of pyruvic acid, γ-methyl-γ-hydroxy-α-ketoglutaric acid, is also active in the glutamine transaminase system (105). The amino acid product appears to be γ-methyl-γ-hydroxyglutamic acid, an amino acid which is structurally similar, and may conceivably be metabolically related to, γ-methyleneglutamic acid. The latter amino acid and its α-keto analogue occur naturally and participate in transamination (106 to 109). Other substituted glutamate and glutamine derivatives, e.g., γ-methylglutamine, γ-methylglutamate, etc. also participate in enzymatic transamination (3), evidence for the natural occurrence of γ-hydroxyglutamic acid (109a) and γ-methyl-γ-hydroxyglutamic acid (109b) has been reported.

Many of the substrates which participate in enzymatic transamination

have been presented in tabular form elsewhere (3). It is very possible that compounds other than these amino and keto acids may also transaminate. Of interest is the report of Gunsalus & Tonzetich (110) that *E. coli* preparations were capable of catalyzing glutamate formation from α-ketoglutarate and adenine, guanine, cytosine, and pyridoxamine. These investigators have apparently not published further studies along these lines. Schein & Brown (111), in a recent abstract, were unable to find clear-cut evidence of transamination of adenine or cytosine with α-ketoglutarate in *E. coli* preparations. On the other hand, Mardashev & Pavlova (112) have reported that rat liver slices catalyze the formation of glycine from glyoxylate (or glycollate) and guanine, adenosine, guanosine, and adenylic acid. It would be most interesting to learn the fate of the purine and pyrimidine derivatives, and whether these compounds undergo change prior to amino group transfer; the nature of such reactions, which may not be "typical" transaminations, may become more apparent when all of the products are identified. It is hoped that further studies of these and related systems will be made.

Clinical application of transaminase.—Recent studies have added serum glutamate-aspartate transaminase to the list of clinically useful enzymes. La Due *et al.* (113) reported that human serum glutamate-aspartate transaminase is significantly elevated following coronary thrombosis associated with myocardial infarction. Enzyme activity was determined by coupling the aspartate-α-ketoglutarate reaction with the malic dehydrogenase system; the reaction was followed by observing the decrease in absorption attributable to DPNH. The relatively weak transaminase activity of normal serum was readily determined by this procedure or by a quantitative paper chromatographic technique (114, 115). In cases of myocardial infarction, the level of glutamate-aspartate transaminase activity of the serum usually rises to two to ten times the normal level within about two days after the onset of clinical symptoms. A return to normal levels occurs within about five days provided that additional areas of the myocardium do not become involved (116 to 120). Although further clinical experience will be necessary for final evaluation of the usefulness of this procedure, the information now available suggests that this technique will prove useful in the diagnosis of myocardial infarction and also in the clinical management of this disorder. Several experimental studies indicate that the heart muscle itself is the source of the increases serum level of enzyme. Thus, in a series of experimental infarctions in dogs, the level of serum enzyme was proportional to the size of the infarct (121, 122). In clinical studies it was found that coronary insufficiency without muscle involvement did not result in increased serum enzyme activity. In view of the wide distribution of glutamate-aspartate transaminase [it has been found even in teeth (123)] it might be expected that damage to other organs would also lead to elevations of the serum enzyme activity. This problem has not yet been extensively attacked; however, Steinberg & Ostrow (124) have reported elevations in serum glutamate-asparate trans-

aminase after abdominal surgery, bowel infarction, and in cases of portal and biliary cirrhosis. It will be of interest to observe the further clinical trial of the glutamate-aspartate transaminase procedure and also to examine serum for the presence of other enzyme activities. It is also possible that cysteine sulfinic acid would offer advantages as a substrate in the test for serum transaminase. Although a number of enzyme systems are known to occur in serum, and several of these have been found to be elevated in certain clinical state (e.g., phosphatase in disease of the liver, prostate, bone; amylase in pancreatitis), it seems probable that only the surface of this field has been explored; further co-operation between enzymologist and clinician might well be productive.

Amino Acid Racemases

Schulze & Bosshard (125) in 1886, were the first to prepare D-isomers of amino acids. They allowed *Penicillium glaucum* to grow on media containing racemic leucine or glutamic acid. The mold preferentially utilized the L-isomer, and they subsequently isolated from the medium leucine or glutamic acid which exhibited optical rotations which were about equal in magnitude but opposite in sign to the corresponding isomers which are known products of acid hydrolysis of protein. From then until relatively recently there was no evidence for the natural occurrence of D-amino acids. It is now evident that a wide variety of D-amino acids occurs in nature, and biochemists have begun to attack the problems involved in their origin and fate.

There is evidence for the occurrence of D-alanine in certain lactic acid bacteria (126, 127); a D-alanine residue is also present in octopine (128, 129, 130). A number of the peptide antibiotics yield D-amino acids on hydrolysis. Thus, evidence for the occurrence of the D-isomers of α-aminoadipic acid (131), α-aminobutyric acid (132), leucine (133, 134), α-methylserine (135, 136), penicillamine (137), phenylalanine (138, 139, 140), and valine (141) has been obtained. In certain instances it is possible that the D-amino acid does not exist in the peptide as such, but is formed on hydrolysis. For example, it now seems probable that L-proline rather than the D-isomer (cf. 142) exists in ergot alkaloids (143) and that the D-allo-isoleucine found in hydrolyzates of actinomycetin is probably formed by epimerization during hydrolysis of L-isoleucine present in the peptide (144). On the other hand, there is little doubt that the capsular material of certain bacteria, including *B. anthracis* and *B. subtilis*, consists of polyglutamic acid predominantly of the D-configuration (145 to 148). D-Glutamic acid also occurs in hydrolyzates of *Lactobacillus arabinosus* (149), and D-aspartic acid is a product of hydrolysis of certain lactic acid bacteria (140). There is evidence for the presence of free D-methionine in *L. arabinosus* (150). Recently, the occurrence of *meso-α*, ϵ-diaminopimelic acid has been reported in a number of microorganisms (151); apparently the LL-form of this amino acid also exists in nature (152). The claim of Kögl & Erxleben (153) that D-glutamic acid and other D-amino acids are present in tumors has been the subject of considerable controversy.

A number of discussions of this problem have appeared (cf. 154, 155), and experimental studies continue to be reported (see, for example, 156).

A number of enzyme systems act on the D-isomers of amino acids, e.g., D-amino acid oxidase (157), D-transaminase (15 to 18), D-cysteine desulfhydrase (158, 159), D-transpeptidase (160), D-asparaginase (161), and D-serine dehydrase (162). In addition, there is evidence for the formation of D-glutamine from D-glutamic acid in the glutamine synthesis system (163), and for the formation of D-kynurenine from D-tryptophan in intact animals (164, 165, 166). It is well known that certain D-amino acid isomers may serve in place of the corresponding L-enantiomorphs in supporting the growth of animals and microorganisms (167, 168). It is probable that in most instances the D-isomer is converted to the α-keto acid analogue by deamination, followed by L-specific amination or transamination. The ability of the α-keto acid analogues of certain amino acids to support growth is consistent with such a mechanism, although direct racemization cannot be excluded in all instances.

The studies which led to the finding of the first amino acid racemase began with a consideration of the growth-stimulating effect of D-alanine on *S. faecalis* (169). Although a number of D-amino acids may serve in place of the corresponding L-enantiomorphs in supporting the growth on microorganisms and of animals, the growth promoting effect of D-alanine for *S. faecalis* was unique in that L-alanine was not effective. Relatively low concentrations of vitamin B_6 sufficed to replace D-alanine in supporting growth and cells grown on media containing D-alanine did not synthesize vitamin B_6, nor was D-alanine a precursor of vitamin B_6. It became evident that vitamin B_6 was needed for the synthesis of D-alanine, and an investigation of the formation of D-alanine in *S. faecalis* by Wood & Gunsalus (170) led to the finding that this organism possessed an enzyme capable of catalyzing the formation of racemic alanine from either isomer. Alanine racemase activity was found in preparations of a wide variety of microorganisms, including *Leuconostoc mesenteroides*, *E. coli*, *B. subtilis*, and *Pseudomonas fluorescens*. Wood & Gunsalus succeeded in obtaining the racemase from sonic extracts of *S. faecalis* and purified the enzyme about four-fold. Pyridoxal phosphate (but not pyridoxamine phosphate) produced striking activation of the partially resolved enzyme. The enzyme exhibited a pH optimum above 8, and catalyzed the formation of racemic alanine from D- or L-alanine at equal rates. No pyruvate could be detected during the reaction, and the evidence suggested a mechanism involving direct racemization. The purified enzyme appeared to be specific for alanine; a number of other amino acids were tested (α-aminobutyric acid, cysteine, serine, leucine, threonine, proline, hydroxyproline, lysine, arginine, histidine, tyrosine, tryptophan, aspartic acid) but they were not racemized under these conditions.

Stewart & Halvorson have investigated the alanine racemase of *Bacillus terminalis* (171). The racemase activity of the spores of this organism was

remarkably resistant to heat inactivation. In contrast with the activity of the vegetative cells, which was virtually destroyed by exposure to 80° C. for 15 min., only 10 per cent of the alanine racemase activity of the spores was lost after heating at 80° C. for 2 hr. Sonic vibration of the spores yielded a preparation in which the racemase remained associated with particulate material, and in this form the activity was again relatively heat stable. Solubilization of the enzyme was accomplished by further sonic oscillation and the soluble enzyme was heat labile. The heat stability of the enzyme therefore appears to be associated in some way with the attachment of the enzyme to a larger particle. Church *et al.* (172) have investigated the germination of spores of aerobic bacteria in the presence of L-alanine; they were unable to find a consistent relationship between germination and the level of activity of alanine racemase. It is obvious that this enzyme system may function so as to provide the cell with either D- or L-alanine. In the case of *B. subtilis* and related organisms, the alanine racemase activity appears to represent the only source of D-amino acids. Once D-alanine is formed, transamination with α-ketoglutarate leads to formation of D-glutamate. It is curious that these organisms, which have a very high requirement for D-glutamic acid, do not apparently possess glutamic acid racemase activity.

Marr and Wilson (172a) found that D-alanine was formed in the oxidation of L-glutamate by washed cells of *Brucella abortus*. A cell-free preparation exhibited alanine racemase activity, whereas the washed cells did not. The authors considered the possibility that D-alanine may be formed by a mechanism other than racemization. When the racemase reaction was carried out in the presence of N^{15}-ammonium ion, there was very little ammonium exchanged; the evidence therefore excludes the participation of ammonium as an intermediate.

Olivard & Snell (173) have utilized the alanine racemase system of *S. faecalis* for a series of studies on vitamin B_6 analogues. Conditions of growth were used under which the concentration of either vitamin B_6 or D-alanine was the growth limiting factor. This system was therefore unique in that the sole use of the vitamin B_6 supplied in the medium was apparently the synthesis of D-alanine. It was found that ω-methylpyridoxal promoted growth in place of pyridoxal, although the analogue was only about 3 to 6 per cent as active as the natural vitamin. ω-Methylpyridoxal phosphate, ω-methylpyridoxamine, and ω-methylpyridoxamine phosphate were also active in supporting growth. In enzymatic studies with a partially purified aporacemase prepared from *S. faecalis*, ω-methylpyridoxal phosphate activated the apoenzyme; the Michaelis constant was 3.5×10^{-6} compared with 4.5×10^{-7} for pyridoxal phosphate. 5-Deoxypyridoxal, 4-nitrosalicylaldehyde, and several other unphosphorylated vitamin B_6 compounds and analogues competitively inhibited activation by pyridoxal phosphate; these findings are consistent with the concept that the phosphate ester group is not necessary for combination with the apoenzyme, although it is obviously needed for formation of an active holoenzyme. However, the presence of a

phosphate ester group increases affinity for the enzyme. Thus, 4-deoxypyridoxine phosphate produced greater inhibition than did 4-deoxypyridoxine itself, when the inhibitors were preincubated with the enzyme. Preincubation of enzyme and coenzyme (or coenzyme analogue) in these studies and in experiments on transaminase (21) is necessary for the occurrence of enzyme-coenzyme (or enzyme-coenzyme analogue) association. Thus 4-deoxypyridoxine phosphate inhibits glutamate-aspartate transaminase (43) and alanine racemase (173) when the inhibitor is incubated with the enzyme prior to addition of coenzyme or substrates. On the other hand, if coenzyme (at saturation levels) is incubated with the enzyme prior to the addition of the inhibitor, no inhibition is observed. The available evidence suggests that the coenzyme is tightly bound to the enzyme, probably by covalent linkages, although the nature of the binding requires further study.

Evidence for the existence of a glutamic acid racemase in *L. arabinosus* was reported independently by Ayengar & Roberts (174) and by Narrod & Wood (175). This organism grows on either D- or L-glutamic acid, and cells grown on L-glutamic acid contain DL-glutamic acid (176, 177). These growth phenomena are consistent with the existence of an enzyme (or enzymes) capable of catalyzing the interconversion of the isomers of glutamic acid. Ayengar *et al.* (178) found that D-glutamic acid was superior to α-ketoglutarate in supporting the growth of *L. arabinosus*. This suggests that interconversion of the isomers of glutamate does not proceed via the α-keto acid analogue, although it is possible that α-ketoglutarate did not enter the cell. Ayengar & Roberts (174) observed conversion of D-glutamic acid to the L-isomer using an acetone powder preparation of the organism. Conversion of L-glutamic acid to D-glutamic acid was also observed; however, in neither type of experiment was the reaction followed to a stage where equal concentrations of the isomers resulted, i.e., a completely racemic product. Such a result was, however, reported by Narrod & Wood (175). Ayengar & Roberts (174) were unable to demonstrate activation by pyridoxal phosphate; on the other hand, Narrod & Wood (175) found that dried *L. arabinosus* cells racemized glutamic acid three times more rapidly in the presence of added pyridoxal phosphate than in its absence. They also found that *L. arabinosus* cells possessed alanine racemase activity. These studies are consistent with the existence of an enzymatic mechanism for the interconversion of the isomers of glutamate in this organism. However, the published data do not prove unequivocally a direct racemization mechanism, although such may indeed be the case. It is possible that the racemization observed could be accomplished, as in the case of the *B. subtilis* system (15, 17), by coupled action of D-transaminase and alanine racemase:

$$\text{D-Glutamic acid} + \text{Pyruvic Acid} \rightleftharpoons \text{D-Alanine} + \alpha\text{-Ketoglutaric acid}$$
$$\text{D-Alanine} \rightleftharpoons \text{L-Alanine}$$
$$\text{L-Alanine} + \alpha\text{-Ketoglutaric Acid} \rightleftharpoons \text{L-Glutamic Acid} + \text{Pyruvic Acid}$$
$$\text{Sum: D-Glutamic Acid} \rightleftharpoons \text{L-Glutamic Acid}$$

Narrod & Wood (175) reported that purified alanine racemase obtained from *L. arabinosus* did not racemize glutamic acid, and Thorne found alanine racemase, but not glutamic acid racemase activity in *B. subtilis* preparations (15, 17). Alanine racemase activity may therefore exist independently of glutamic acid racemase activity. The demonstration of glutamic acid racemase free from alanine racemase activity remains to be accomplished.

Shockman & Toennies (150, 179) have made some interesting observations on *S. faecalis*, which suggest that this organism possesses a mechanism for the interconversion of D- and L-methionine. They observed that although D-methionine does not support the growth of *S. faecalis*, that this isomer could be utilized in the presence of L-methionine during the postlogarithmic phase of growth. The organism may, therefore, utilize L- or DL-methionine, but not D-methionine; it would be fallacious in this case to assume from the inactivity of the D-isomer, that only half of the racemic form was active. This type of assumption is often made in studies in which racemic amino acids are used. Shockman & Toennis also observed that only about 67 per cent of the L-methionine supplied in the growth medium could be accounted for in the cell hydrolyzate and supernatant solution. It had been possible to account for virtually all of each of six other amino acids (histidine, isoleucine, leucine, lysine, threonine, and valine) in similar experiments (180). About 20 per cent of the original L-methionine could be accounted for as D-methionine or D-methionine sulfoxide in the cell supernatant solution. Conversion to the sulfoxide was ascribed to nonenzymatic oxidation. The authors suggested the possible existence of a methionine racemase to explain these findings; enzymatic studies on this organism have not as yet been published. On the other hand, if the organism possesses methione racemase activity, it might be expected to grow on D-methionine in the absence of the L-isomer.

Kallio & Larson (181) have carried out enzymatic studies of methionine racemase using strains of *Pseudomonas* isolated by enrichment culture with racemic methionine. Using a fraction obtained by ammonium sulfate precipitation, they observed conversion of both D- and L-isomers to the racemate. After purification by adsorption on and elution from calcium phosphate gel, addition of pyridoxal phosphate was required for maximal activity. The racemase exhibited optimal activity between pH values of 8 and 9. The possibility that the observed racemization of methionine was attributable to coupled alanine racemase and D-transamination seems ununlikely because addition of pyruvate or α-keto-γ-methiolbutyrate did not affect the reaction. It would also be important to determine whether these preparations exhibit alanine racemase activity.

Hoare & Work (182) have recently described the occurrence of a unique amino acid racemase present in certain bacteria. The discovery of α,ϵ-diaminopimelic acid in *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis* (151, 183, 184) was followed shortly by the finding of a decarboxylase system in several organisms which converted α,ϵ-diaminopimelic acid

to L-lysine and carbon dioxide (185). The preparation of the three possible stereoisomers of α,ϵ-diaminopimelic acid (186) gave considerable impetus to this research, for it could subsequently be shown that the isomer originally isolated was of the *meso* configuration (187). The LL-isomer was also found in certain organisms (152). Study of the decarboxylase system revealed that the DD-isomer was not attacked and that both *meso* and LL-isomers were decarboxylated to L-lysine (or, in the presence of L-lysine decarboxylase, to cadaverine). Acetone-dried preparations of *Enterobacteriacea* invariably decarboxylated the *meso* form more rapidly than the LL-isomer, and there was occasionally a lag period in the decarboxylation of the latter isomer (182). When the decarboxylase was purified (from *Aerobacter aerogenes*), the LL-isomer was decarboxylated at only about 2 per cent of the rate observed with the *meso* isomer. This result suggested that either there were separate decarboxylases for the LL- and *meso* isomers, or that a system existed for the conversion of the LL- to the *meso* isomer. The latter interpretation was supported by the finding that a lysineless mutant of *E. coli* was capable of interconverting the *meso* and LL forms. The reaction has thus far only been observed qualitatively by paper chromatography, taking advantage of the fact that the *meso* and LL-isomers behave differently with a solvent consisting of methanol, water, and pyridine (80:20:4) (188). Since the DD-isomer is not active in the racemase system, it may be concluded that the enzyme racemizes only one asymmetric center. Hoare & Work have reported that equilibrium is reached starting with either the LL or the *meso* form when about equivalent concentrations of both isomers are present. The pH optimum of the racemase is about 8.5; the enzyme loses activity on dialysis and may be reactivated by addition of thiols. Inhibition by hydroxylamine, semicarbazide, and *p*-chloromercuribenzoate (reversed by glutathione) was also noted. As yet, there is no evidence for the participation of vitamin B_6 in this reaction. These enzymatic reactions of diaminopimelic acid may be summarized as follows:

$$\text{LL-Diaminopimelic Acid} \rightleftharpoons \textit{meso}\text{-Diaminopimelic Acid}$$
$$\textit{meso}\text{-Diaminopimelic Acid} \rightarrow \text{L-Lysine} + CO_2$$
$$\text{L-Lysine} \rightarrow \text{Cadaverine} + CO_2$$

In addition to the discovery of the natural occurrence of two isomers of a new amino acid this research has revealed at least three findings of interest: (*a*) an amino acid decarboxylase which acts on a D-asymmetric center, (*b*) a racemase which acts on only one asymmetric center of an amino acid possessing two asymmetric carbon atoms, and (*c*) paper chromatographic separation of the isomers of an aliphatic amino acid.

Evidence for a threonine racemase in *E. coli* has been reported by Amos (189). A cell-free extract of the dried cells was incubated with D-threonine, adenosinetriphosphate (or adenosinemonophosphate), phosphate buffer (pH 7.8), and *Colstridium welchii* L-threonine deaminase. The deaminase was inactive toward D- or L-allothreonine and D-threonine. Under these conditions, about 10 μM of ammonia were formed from 50 μM of D-threonine.

The formation of L-threonine was also suggested by assay with an L-threonine-requiring mutant of *E. coli*. Pyridoxal phosphate did not affect the rate of the reaction, whereas the presence of *C. welchii* L-threonine deaminase appeared to accelerate the reaction. Evidence consistent with the conversion of L-threonine to D-threonine was obtained using the same system by following the rate of L-threonine disappearance. This reaction, which has been only partially characterized, may be a complicated one since the conversion of L-threonine to D-threonine requires inversion of both asymmetric carbon atoms. The requirement for adenosinetriphosphate (or adenosinemonophosphate) is unusual in terms of other racemases which have thus far been investigated. Further study of this system should prove interesting.

Snell and collaborators have advanced a general mechanism for vitamin B_6-catalyzed reactions, in which racemization is explained in terms of Schiff base formation between pyridoxal and amino acid, and reversible formation of a double bond between the α-carbon atom of the amino acid and the nitrogen atom (23). This mechanism is supported by nonenzymatic studies in which the racemization of several amino acids was observed at 100° C. in the presence of pyridoxal and metal ions (Cu^{++}, Fe^{++}, Al^{+++}) (190). This proposal, as in the case of transamination, is eminently logical; however, at this time vitamin B_6 has not shown to participate in all amino acid racemase systems, and it remains to be proven that this mechanism holds for the enzymatic racemization reactions of amino acids.

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PROTEOLYTIC ENZYMES[1,2]

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Exopeptidases

Leucine aminopeptidase.—Most of the earlier studies on the purification and properties of this enzyme have referred to the leucine aminopeptidase of intestinal mucosa. The high activity exhibited by extracts of swine kidney toward L-leucinamide (1) makes this tissue a more suitable source of the enzyme. Spackman *et al.* (2) have developed a valuable method for the purification of swine kidney leucine aminopeptidase by treatment of the tissue with acetone, fractionation with ammonium sulfate, precipitation with $MgCl_2$, heating to 70°C., acetone fractionation, aging, and preparative electrophoresis on filter paper. The final preparation was 1600 times as active per mg. of protein as a crude aqueous extract of swine kidney. It migrated as a single component in the Tiselius cell at pH 8.5, and sedimentation studies at this pH showed a single boundary. Extrapolation to zero protein concentration gave a value of s_{20} of 12.6S; this was interpreted to indicate a particle weight of about 300,000. However, since Mg^{++} was present to stabilize the enzyme, the possibility exists that this value may refer to a product of aggregation. The ultraviolet absorption spectrum showed a typical maximum at about 280 mμ; there was no evidence for the presence of nucleotides, as has been suggested in other studies (3) with swine kidney peptidases. Chromatographic analysis of an acid hydrolysate of the highly purified preparation of Spackman *et al.* accounted for about 97.5 per cent of the protein N as amino acids and ammonia; especially noteworthy is the high leucine content (8.8 per cent).

Smith & Spackman (4) have examined the activation behavior of their highly purified swine kidney leucine aminopeptidase and have confirmed and extended the earlier observations on the enzyme from swine intestinal mucosa (5). In its action on L-leucinamide, the swine kidney enzyme requires activation by Mn^{++} or Mg^{++}; other divalent cations either have no effect or are inhibitory. An extensive investigation of the specificity of the swine kidney enzyme (4) has also confirmed earlier results which showed that L-leucine methyl ester is not a substrate, and that various amino acid amides, dipeptides, and dipeptide amides are cleaved by the enzyme in the pH range 8 to 9. The authors have reaffirmed the earlier conclusion that leucine aminopeptidase acts upon L-leucinamide by chelation of the metal ion by two

[1] The survey of the literature pertaining to this review was completed in October, 1955.

[2] The following abbreviations are used: ADP for adenosinediphosphate; AMP for adenosinemonophosphate; BAL for British antilewisite (2,3-dimercaptopropanol); ATP for adenosinetriphosphate; DENP for diethyl-*p*-nitrophosphonate; GSH for reduced glutathione; TMV for tobacco mosaic virus; and DP for diisopropylphosphoryl.

groups of the enzyme protein and by the α-amino nitrogen and the imide nitrogen of the substrate; a third group of the protein is considered to interact with the side chain of the substrate.

Other recent studies on aminopeptidases are those of Kirshner & Pritham (6), of Gomori (7), and of Green *et al.* (8). In the latter two papers, methods are described for the use of glycyl-β-naphthylamine or of L-leucyl-β-naphthylamine as chromogenic substrates for the determination of aminopeptidase activity in tissue extracts.

Tripeptidase.—Davis & Smith (9) have confirmed the earlier conclusions (10) in regard to the requirement of this enzyme for a specified chain length in its substrates. These workers have found that tripeptidase from horse erythrocytes hydrolyzes glycyl-δ-aminovaleric acid, in which the spatial separation between the free amino and carboxyl groups is similar to that present in tripeptides composed of α-amino acids. A study of tripeptidase activity of synovial fluid from patients with arthritic disease has been reported (11).

Iminodipeptidase.—Davis & Smith (12) have reported that a purified preparation of this enzyme (from swine kidney) hydrolyzes hydroxy-L-prolylglycine, allohydroxy-L-prolylglycine, and methoxy-L-prolylglycine.

Carboxypeptidases.—Yanari & Mitz (13) have examined the action of pancreatic carboxypeptidase on dipeptides, and believe that to serve as substrates, they must have an uncharged α-amino group. At pH 9, dipeptides are effective competitive inhibitors of carboxypeptidase. Fu *et al.* (14) have studied the effect of the presence of optically active acyl groups (α-chloropropionyl, carbobenzoxyalanyl) on the cleavage, by pancreatic carboxypeptidase, of the C-terminal bond in acyl-L-phenylalanine and acyl-L-tyrosine compounds. Relatively little difference was found with the isomeric α-chloropropionyl derivatives, but the carbobenzoxy-L-alanyl compounds were hydrolyzed more rapidly than were the corresponding D-compounds. These findings were compared with those obtained with a kidney carboxypeptidase preparation (renal acylase I), which acts much more rapidly on substrates having L-acyl substituents than on those having D-acyl substituents. With the α-chloropropionyl derivatives, this difference became more marked as the size of the side chain of the terminal amino acid increased from glycine to L-norvaline. The action of acylase I on some acetyl-L- and D-amino acids is accelerated by the addition of cobaltous ions (15).

Other studies on exopeptidases.—Doyle (16) has described an interesting investigation on the distribution of peptidase activity in the lymphatic tissue of the rabbit and has demonstrated the presence of a labile peptidase (substrate, L-alanylglycine) in macrophages and in the extracellular fluid. Work on the peptidase activity of lymphatic tissue has also been reported by Gullino (17). Fleischer (18) has described the action of peptidases present in normal human leucocytes.

The presence, in fish muscle, of a peptidase that hydrolyzes anserine to 1-methyl-L-histidine and β-alanine has been reported (19). This enzyme appears to be activated by Zn^{++} ions.

Endopeptidases

Pepsin.—In confirmation of the work of Flavin (20), de Verdier (21) has reported that partial hydrolysis of pepsin yields phospho-L-serine and a phosphopeptide composed of serine, threonine, glutamic acid, and phosphoric acid.

Christensen (22) has shown that the pH dependence of the action of crystalline pepsin is different for the native and denatured forms of each of several proteins. For example, whereas the pH optimum for the hydrolysis of native ovalbumin is about 1.0, and the rate is rather slow at pH values more alkaline than pH 2, the denatured protein is readily hydrolyzed within a wide pH range (1 to 4). These results support the view that the initial step in the peptic hydrolysis of a native protein involves an unfolding of the peptide chains of the substrate molecule. The action of pepsin on synthetic peptides hydrolyzed by the enzyme at pH values near 4 thus resembles the action on denatured proteins. Baker (23) has extended the study of the peptic hydrolysis of the synthetic substrates acetyl-L-phenylalanyl-L-tyrosine and acetyl-L-tyrosyl-L-tyrosine and has described the kinetics of the reaction. Laidler (24) has extended his studies on the pH dependence of pepsin action. Masch & Huchting (25) note that the pH optimum of pepsin action ranges between 1.8 and 3.8, and depends on the nature of the substrate and of the acid anion; they believe it to be unnecessary to postulate a gastric cathepsin acting at pH 4 (26).

Bock (27) has described a method for the routine determination of pepsin in gastric juice, using dried human serum as the substrate. Marini & Levey (28) have reported on the effect of pepsin inhibitors on the milk clotting activity of crystalline pepsin.

Chymotrypsin.—Further progress has been made in the laboratories of Neurath and of Desnuelle to elucidate the sequence of reactions in the conversion of chymotrypsinogen to π-chymotrypsin, δ-chymotrypsin, and α-chymotrypsin. Much of this work, which ranks among the most significant recent contributions in the field, has been reviewed by Schwert (29). During the period covered by the present review, the following additional information has been reported. In contrast to the earlier view that bovine chymotrypsinogen-α has no free α-amino groups, Bettelheim (30) has shown that in this protein, one of the cystine residues has a free α-NH_2 group, i.e., there is one N-terminal half-cystine residue. It had been shown by Jacobsen (30a) that the rapid activation of chymotrypsinogen-α by trypsin leads to the formation of two products, identified as π-chymotrypsin and δ-chymotrypsin. π-Chymotrypsin is now reported (31) to have the same electrophoretic mobility at pH 4.97 as does chymotrypsinogen, and to contain one residue each of N-terminal isoleucine and half-cystine, but no C-terminal amino acid residue that can be removed readily by carboxypeptidase. δ-Chymotrypsin has a lower electrophoretic mobility at pH 4.97, the same N-terminal amino acid groups as π-chymotrypsin, and in addition a C-terminal leucine residue susceptible to carboxypeptidase action. The conversion of π-chymotrypsin to

δ-chymotrypsin appears to be an autolytic process since it is inhibited by β-phenylpropionate (which inhibits chymotrypsin) but not by soy bean trypsin inhibitor. The change in the electrophoretic behavior suggested that a basic peptide was split off during the $\pi \rightarrow \delta$ conversion, and this has been established by the demonstration that serylarginine is formed in stoichiometrically significant amounts (32, 33).

The currently available data have been interpreted by both the Neurath group and the Desnuelle group to indicate that the conversion of chymotrypsinogen to δ-chymotrypsin involves the successive action of trypsin and chymotrypsin on the peptide sequence —Leu. Ser. Arg. Ileu—. Trypsin attacks the arginyl-isoleucyl bond to form π-chymotrypsin, and this is followed by autolysis of the leucyl-seryl bond. Earlier work had shown that α-chymotrypsin has N-terminal alanyl and isoleucyl groups, and it is likely that this form of the enzyme arises by the autolytic cleavage of a tyrosyl-alanyl bond in the peptide chain.

Since the tryptic cleavage of only one peptide bond of chymotrypsinogen leads to the formation of an active π-chymotrypsin, the further study of the process is likely to yield important new knowledge about the structural basis of the enzymic activity. Rupley, Dreyer & Neurath (34) have shown that, during the activation of chymotrypsinogen, the optical rotation shifts to more positive values, and the rate of this change is the same as the rate of appearance of enzymic activity. These authors note that this change in rotation is opposite in direction from that observed for the denaturation of proteins. An electrophoretic study of the activation of chymotrypsinogen has been reported by Egan (35).

Schaffer *et al.* have continued their studies on the products obtained upon hydrolysis of diisopropylphosphoryl-chymotrypsin (DP-chymotrypsin). In earlier reports (36) the identification of phosphoserine and phosphoserylglycine was described. On hydrolysis of DP^{32}-chymotrypsin with 12 *N* HCl at 37°C. for three days, followed by fractionation on Dowex-50 columns, the following radioactive peptides were identified: aspartylphosphoserine, phosphoserylglycine, and aspartylphosphoserylglycine (37). The authors suggest that the aspartyl residues may be present in the protein as the corresponding β-amide, since other fractions in the chromatographic separation contained asparaginyl residues.

A study of the hydrolysis of DP-chymotrypsin by a mixture of pancreatic enzymes (Cotazym) has been reported by Oosterbaan *et al.* (38). A phosphopeptide was isolated and the component amino acids were found to be leucine, aspartic acid, proline, serine, and glycine.

The important studies of Hartley & Kilby (39) on the action of chymotrypsin on acetyl-*p*-nitrophenol led them to the conclusion that an acetylchymotrypsin was formed. This has been followed by the significant achievement of Balls & Aldrich (40) in the isolation of this postulated intermediate. These investigators took advantage of the fact that at pH 5 to 6, free *p*-nitrophenol is rapidly released and the chymotrypsin is inactive toward L-tyrosine

ethyl ester. At pH 7.2, the inhibited chymotrypsin readily regains its original activity toward the synthetic substrate and in the clotting of milk. Since the inhibited enzyme reacts with hydroxylamine to form one mole of hydroxamic acid per mole of protein, it is inferred that a monoacetyl-chymotrypsin was formed in the reaction at pH 5. That the acetylation involves a group concerned with the enzymic activity of chymotrypsin is indicated not only by the inhibition, but also by the fact that chymotrypsinogen, denatured chymotrypsin, and DP-chymotrypsin fail to cause the rapid liberation of *p*-nitrophenol from acetyl-*p*-nitrophenol at pH 5. The nature of the chemical group in chymotrypsin that is acetylated is still unclear, but obviously presents a question of great importance. Balls & Aldrich raise the possibility that it may be a sulfhydryl group (41), and Hartley (42) has suggested the imidazolyl group of a histidine residue as the site of attack. Both thiolesters and acylimidazoles are known to be reactive acylating agents. Since DP-chymotrypsin is inactive in catalyzing the release of *p*-nitrophenol, it would appear that the diisopropylphosphoryl group may be attached to the same group in the protein as that which is acetylated. If this is so, the possibility must be considered that the phosphoserine isolated from hydrolysates of DP-chymotrypsin may be formed by acyl migration in the course of acid hydrolysis. Michel (43) has studied the kinetics of the reaction of chymotrypsin and of trypsin with diisopropylfluorophosphate.

A further contribution to the problem of the "active center" of α-chymotrypsin is the study of Wood & Balls (44), who have oxidized the enzyme by H_2O_2 in the presence of horse radish peroxidase, and have shown that the crystalline protein which was isolated had lost 1 mole of tryptophan and had one-half of its original activity toward L-tyrosine ethyl ester.

Foster & Niemann (45) have discussed critically the assumptions and experimental limitations that must be taken into account in the determination of the values of K_m and of k_3 for the hydrolysis of synthetic substrates by chymotrypsin, and have re-evaluated these constants for a large number of substrates studied previously. They note that a relationship exists between the magnitude of k_3 and the refractivity of the R′ group in substrates of the general formula $R'CONH.CH(CH_2.C_6H_4OH)CONH_2$. A critical re-evaluation of the inhibition constants (K_I) for a large series of acylamino acid anions and of acyl-D-amino acid amides has also been reported. These results have led Foster, Shine & Niemann (46) to suggest that the modes of combination of a sensitive acyl-L-amino acid amide with the enzyme is different from that for an inhibitory acyl-D-amino acid amide. Another report from the same laboratory (47) summarizes revised values of K_I for a variety of other competitive inhibitors of chymotrypsin. A study of the effect of change in pH on the values of K_I for a series of charged and uncharged competitive inhibitors of chymotrypsin has been performed by Foster & Niemann (48), who suggest that the decreased affinity of the enzyme at pH 6.9 for some negatively charged competitive inhibitors, as compared with the behavior at pH 7.9, is a consequence of the development of a negative charge in the environment

of the catalytic region of the enzyme molecule. Shine & Niemann (49) have reported that, in the action of chymotrypsin on chloroacetyl-L-tyrosinamide, the initial rate is increased by addition of NaCl or of KCl. Although the value of K_m is unchanged by this addition, the magnitude of k_3 is increased by the amount given by the relation log $(k_3/k_3^\circ) = 0.3\sqrt{M}$, where M is the molarity of the solution with respect to NaCl or KCl.

Vaslow (50) has studied the kinetics of the chymotrypsin-catalyzed exchange of O^{18} for O^{16} in N-acetyl-3,5-dibromo-L-tyrosine in H_2O^{18}. Satisfactory agreement was obtained between the Michaelis constant, determined kinetically, and the equilibrium constant of the binding of the tyrosine derivative by chymotrypsin. The rate of turnover for the exchange reaction is of the same order of magnitude as that found for the chymotrypsin-catalyzed hydrolysis of N-acetyl-3,5-dibromo-L-tyrosinamide, and of other susceptible amides.

Bender & Turnquest (51) have concluded, from a comparison of the values for k_3 for the hydrolysis of ethyl esters of several α-substituted-β-phenylpropionic acids by alkali, by acid, and by chymotrypsin, that no correlation was possible between the nonenzymic and enzymic hydrolysis, so far as the breakdown of the enzyme-substrate complex was concerned. These investigators suggest that hydrogen bonding of the substrate to a water molecule may be important in determining the rate of decomposition of enzyme-substrate complex.

Doherty (52) has given a detailed report of the action of chymotrypsin on the ethyl ester of 5-(*p*-hydroxyphenyl)-3-ketovaleric acid to form *p*-hydroxyphenylpropionic acid. At the optimal pH of 8, and at enzyme concentrations of 0.5 to 2 mg. of N per ml., $K_m = 7 \times 10^{-2}$M and $k_3 = 2.5 \times 10^{-3}$M/min./mg. of protein N/ml. This important discovery of a substrate cleaved by chymotrypsin at a carbon-carbon bond further extends the range of the specificity of chymotrypsin.

Wu & Laskowski (53) have described the action of several natural trypsin inhibitors on α-chymotrypsin and chymotrypsin B. A preliminary report of this work was reviewed by Schwert (29).

Holley (54) has suggested the application of chymotrypsin as an aid in peptide synthesis by the use of the benzoyl-L-phenylalanyl group to block the α-amino group of the desired peptide, and the enzymic removal of this group after the coupling reaction. The limitations of this technique are pointed out by the author.

Schwert & Takenaka (55) have described a spectrophotometric method for the determination of chymotrypsin, with tyrosine ethyl ester as the substrate. The method depends on the difference in absorption at 233.5 mμ between the ester and the corresponding carboxylate ion. A similar procedure has also been devised for the measurement of the action of trypsin on benzoylarginine ethyl ester.

Trypsin.—The nature of the peptide liberated from trypsinogen when it is activated by trypsin has been established more definitely in work by the laboratories of Neurath and of Desnuelle. All the available evidence indicates

the peptide to be Val. $(Asp)_4$.Lys (56, 57), which is cleaved from the N-terminal end of the trypsinogen by the scission of a lysyl-isoleucyl bond.

Cunningham (58) has conducted measurements of the diffusion constant of diisopropylphosphoryl-trypsin (DP-trypsin); these data, in conjunction with earlier sedimentation studies, have given a value of 23,800 for the particle weight of the protein. The isoelectric point was found to be 10.5.

Gutfreund (59) has examined the effect of pH on the rate of hydrolysis of benzoyl-L-arginine ethyl ester by crystalline trypsin and has interpreted the results to indicate that an uncharged imidazolyl group of histidine is essential for the catalysis. Bernhard (60) has measured K_m and k_3 for the tryptic hydrolysis of benzoyl-L-argininamide by a formol titration method, and has reported values of 3.1×10^{-3}M and 0.043 sec.$^{-1}$ respectively, at pH 7.6 and 25°C. He has also compared the values of K_m for benzoyl-L-argininamide with the value of K_I when the amide acts as a competitive inhibitor of the tryptic hydrolysis of benzoyl-L-arginine ethyl ester (61). The two constants were found to be equal, and this result was interpreted to indicate that the rate of dissociation of the enzyme-substrate complex to free enzyme and reactant is much greater than the rate of the decomposition of the complex (k_3). Hence, for the tryptic hydrolysis of benzoyl-L-argininamide, $K_m = k_2/k_1$.

Viswanatha & Liener (62) have shown that, at a high concentration of urea (6.6 to 8.3 *M*), trypsin retains its proteolytic activity, whereas at urea concentrations of 3.3 to 5.0 *M*, trypsin activity is rapidly lost at pH 7.6. The latter treatment is accompanied by the release of trichloroacetic acid-soluble products, suggesting enhanced autolysis of the enzyme. The presence of Ca^{++}, of decylbenzene sulfonate, or of substrate (casein) protected the enzyme from inactivation by urea. Subsequent studies (63) have shown that the active trypsin at high urea concentrations differs from untreated trypsin in the response to cysteine and to diethyl-*p*-nitrophosphonate (DENP). The activity of urea-treated trypsin is depressed by 0.005 M cysteine, whereas untreated trypsin is unaffected; DENP does not inhibit the urea-treated trypsin, although *p*-nitrophenol is liberated. The authors suggest that the site of DENP action may not be the same as the catalytic region of the enzyme. The possibility must also be considered that the diethylphosphoryl derivative of the urea-treated trypsin may be much less stable to hydrolysis than that of the untreated trypsin. A study of the effect of synthetic anionic detergents on trypsin has been reported (64).

A detailed report of the studies of Davie & Neurath on the terminal groups of the soy bean trypsin inhibitor has been published (65); this work was reviewed last year (29). To the known natural inhibitors of trypsin may be added the materials obtained in crystalline form from the Indian field bean and the double bean (66); a trypsin inhibitor has been found in swine colostrum (67). The partial purification of a trypsin inhibitor from normal urine has been reported (68). Jacobsson (69) has provided evidence in favor of the view that urea can cause the dissociation of the complex formed between trypsin and the soy bean inhibitor.

Ferguson & Løvtrup (70) have compared the relative usefulness of casein

and nitrocasein as substrates of crystalline and crude trypsin preparations, and have concluded that the casein method is the more sensitive.

The acylation of some of the amino groups of crystalline trypsin by treatment with various acid anhydrides (propionic, butyric, citraconic, and itaconic) has been described (71). The specific activity of the resulting derivatives is comparable to that of the untreated enzyme, although the pH optima toward casein and benzoyl-L-arginine ethyl ester were at a somewhat more alkaline pH value.

Grant & Robbins have reported, in a preliminary communication (72), that hog pancreas contains a proteinase different from trypsin or α-chymotrypsin. A partially purified preparation of the new enzyme (pankrin) exhibited more rapid degradation of protein substrates than did either of the known pancreatic proteinases. Pankrin appears to hydrolyze both toluenesulfonyl-L-arginine methyl ester and acetyl-L-tyrosine ethyl ester. The authors claim to have ruled out the possibility that these effects are a consequence of the synergistic action of a mixture of proteinases.

Bresler *et al.* (73) have reported that autolysis of crystalline trypsin at pH 9.15 leads to release of active fragments that do not sediment upon high speed centrifugation. McDonald (74) has examined the effects of changes in enzyme concentration, of the solvent, of pH, and of temperature on the inactivation of dilute solutions of crystalline trypsin by x-radiation.

Minganti (75) has described the action of trypsin and other proteinases (chymotrypsin, papain) on the oocytes and mature egg of the sea urchin (*Psammechinus miliaris*), and Moore (76) has reported that sea urchin embryos which develop in sea water containing trypsin show marked abnormalities.

Other studies in which trypsin has been employed include experiments on the synergistic action of lysozyme and trypsin in bacteriolysis (77), the inhibition of edema by treatment with trypsin or chymotrypsin (78, 79), and the antiphlogistic effect of trypsin in normal and adrenalectomized rats (80).

Papain.—Johnston (81) has reported the hydrolysis of thiol esters by activated papain; hippuryl ethanethiol and hippuryl 2-propanethiol are hydrolyzed at approximately the same rate, which is more rapid than that for hippuric acid ethyl ester or hippurylamide.

Cathepsins.—Recent work by Wiggans *et al.* (82) has shown that cathepsin C, a proteinase from beef spleen (83), catalyzes the hydrolysis of ester linkages as well as amide bonds of suitable synthetic substrates. Glycyl-L-phenylalanine ethyl ester, glycyl-L-tyrosine ethyl ester and glycylglycine ethyl ester were hydrolyzed at pH 5, whereas β-alanyl-L-phenylalanine ethyl ester, β-alanyl-L-tyrosine ethyl ester, glycine ethyl ester, L-phenylalanine ethyl ester and L-tyrosine ethyl ester were resistant to enzyme action. Glycylglycinamide is a poor substrate for cathepsin C; however, the corresponding ethyl ester is hydrolyzed as readily as is glycyl-L-phenylalanine ethyl ester or glycyl-L-tyrosine ethyl ester. Cathepsin C appears to be restricted to the hydrolysis of amide or ester derivatives of dipeptides composed of α-amino acid residues. Although the specificity of cathepsin C

resembles that of α-chymotrypsin in some respects, it differs from that of chymotrypsin in the requirement for a free α-amino group at a definite distance from the carbonyl group of the sensitive ester or amide linkage. At pH values near 7.5, insoluble precipitates were formed when cathepsin C was incubated with glycyl-L-tyrosine ethyl ester, with glycyl-L-phenylalanine ethyl ester, or with glycylglycine ethyl ester. Although the nature of these precipitates was not determined, it has been suggested that they are long-chain peptides formed by a polymerization of the dipeptide esters through a series of successive replacement reactions similar to those shown to occur when glycyl-L-phenylalaninamide is incubated with cathepsin C at pH 7.5 (84). Two additional dipeptide amides have been found to undergo polymerization when incubated with cathepsin C at pH 7.5; they are L-seryl-L-phenylalaninamide and L-seryl-L-tyrosinamide. At pH 5.0, these compounds are readily deamidated by hydrolysis (82).

Dannenberg & Smith (85, 86) have shown that extracts of beef lung contain, in addition to several exopeptidases (leucine aminopeptidase, tripeptidase, glycylglycine dipeptidase, prolidase, iminodipeptidase), proteinase activity, toward hemoglobin, ascribed to two enzymes with optima at pH 4 (Proteinase I) and at 8.4 (Proteinase II). Proteinase I was partially purified by the preparation of an acetone powder, fractionation with ammonium sulfate, and adsorption on calcium phosphate gel. This enzyme was inhibited by *p*-chloromercuribenzoate and iodoacetamide, but not by Versene (tetrasodium salt of ethylenediaminotetraacetic acid) and diisopropylfluorophosphate; the partially purified Proteinase I was activated by cysteine. Although none of the synthetic substrates tested with Proteinase I were hydrolyzed, the oxidized B chain of insulin was found to be cleaved in a manner resembling the action of pepsin. Preparations of Proteinase I also catalyze the polymerization of several amino acid esters.

Vaughan (87) has effected a partial purification of the proteolytic enzyme (of rat liver) that causes inactivation of insulin at pH 7.5, and named insulinase. The enzyme preparation hydrolyzes peptide bonds of intact insulin, and of the A and B chains of oxidized insulin. Tomizawa *et al.* (88) and Mirsky *et al.* (89) have examined the action of rat liver extracts on I^{131}-labeled insulin.

Laver & Trikojus (90) have effected a one-hundred and fiftyfold purification of the proteinase activity of the swine thyroid gland. In the course of these studies, the earlier reports of Weiss (91) on the intracellular distribution of peptidase and proteinase activity in thyroid tissue were confirmed. The formation of thyroxine, monoiodotyrosine, and diiodotyrosine in an autolysate of rat thyroid has been ascribed to the breakdown of thyroglobuin by a proteolytic enzyme having a pH optimum at about pH 4.9 (92).

Goetze & Rapoport (93) have reported the presence, in rabbit erythrocytes, of a cathepsin with a pH optimum of 3.5; its activity has been found to be greatest in bone marrow and greater in reticulocytes than in mature erythrocytes. Unlike a similar enzyme investigated by Morrison & Neurath (94), this cathepsin is inhibited by ascorbic acid and Zn^{++}. Leucocytes and lym-

phocytes exhibit catheptic activity with a pH optimum of 4.5 (95). The digestion of caseous tubercles by a leucocytic proteinase has been reported by Weiss & Tabachnik (96). Hess *et al.* (97) have presented evidence for the presence in bovine palatine tonsils, of a proteinase having maximal activity at pH 3 toward bovine serum albumin. The suggestion has been made that the enzyme is similar to cathepsin C.

An interesting investigation on the intracellular distribution of catheptic activity of rat liver tissue has been performed by de Duve *et al.* (98). By the use of a method described by Gianetto & de Duve (99), the proteinase activity was found to be largely localized in the mitochondrial fractions, from which the enzyme activity is released by freezing and thawing or exposure to hypotonic media. In many respects, the distribution of the catheptic activity toward hemoglobin resembles that of acid phosphatase, ribonuclease, and deoxyribonuclease. Siebert (100) has reported the presence, in swine kidney nuclei, of a proteinase that hydrolyzes several proteins at about pH 8.

Ungar & Damgaard (101) have interpreted their studies on the effect of thermal injury to skin as indicating the conversion of an inactive precursor, present in normal tissue, into an active proteinase. The same authors report that upon β-irradiation of slices of rat skin, proteolysis is inhibited (102). Fodor *et al.* (103) have reported that, during the regression of Flexner-Jobling carcinomas, the catheptic activity of the tumors increases markedly. Deutsch & Dinz (104) have described the proteolytic activity of some snake venoms.

Thrombin.—The presence of soy bean trypsin inhibitor suppresses the formation of thrombin from prothrombin in systems activated by either citrate (105) or thrombokinase (106). Dextran has also been shown to inhibit the activation of prothrombin (107). Compounds known to alter the disulfide linkages of proteins (e.g., cysteine, glutathione, BAL, thioglycollic acid) caused the inhibition of the clotting activity of purified prothrombin, whereas compounds that affect sulfhydryl groups (e.g., iodoacetate, *p*-chloromercuribenzoate) had no effect (108).

Since *p*-toluenesulfonyl-L-arginine methyl ester is hydrolyzed by thrombin (109), this substrate may be used for the quantitative assay of prothrombin in plasma (110). This method has several advantages over the older clotting techniques.

Prothrombin and thrombin have been subjected to zone electrophoresis by Lanchantin *et al.* (111). Rasmussen (112) has described the purification of thrombin by means of chromatography on Amberlite IRC-50 columns. The most active fractions showed a fiftyfold purification. Sherry *et al.* (113) have reviewed the status of thrombin as a proteolytic enzyme.

Donnelly *et al.* (114) have demonstrated the reversibility of the process: fibrinogen$\rightleftharpoons$fibrin monomer+fibrinopeptide, catalyzed by thrombin. Sedimentation, flow birefringence, and light-scattering measurements at several pH values showed that, at pH 5.3 and in the presence of molar NaBr, fibrin is converted to fibrin monomer; at pH 6.1, the polymeric form predominates. By the use of toluenesulfonyl-L-arginine methyl ester as a competitive in-

hibitor of thrombin (109), Donnelly *et al.* (115) were able to determine the equilibrium constants in the above reaction. Tinoco & Ferry (116) have offered evidence for the view that the site at which fibrinogen is attacked by thrombin is on one side of the rod-shaped fibrinogen molecule, midway between the ends. Biezunski *et al.* (117) have reported that poly-L-lysine accelerates the enzymic conversion of fibrinogen to fibrin.

Plasmin (fibrinolysin).—Kline (118) has been unable to confirm the report (119) that the fibrinolytic and proteolytic activities of purified plasminogen could be separated; he ascribes the failure to find proteolytic activity in fibrinolysin preparations to high dilution. Arginine methyl ester, arginine ethyl ester, and lysine ethyl ester protect plasmin from destruction when plasminogen is activated by streptokinase (118); at pH 8 the arginine esters gave rise to precipitates from which the enzyme could not be eluted. Similar results were obtained when cathepsin C acted on dipeptide amides to form polymeric peptides (120). Paly & Kline (121) believe that the spontaneous proteolytic activity of the euglobulin fraction of plasma is attributable to an enzyme different from plasmin. Schulman (122) has examined the action of bovine fibrinolysin on fibrinogen by means of ultracentrifugation. Further evidence has been presented to show that plasmin and trypsin, though similar in some respects, are not identical (123).

The studies of Troll & Sherry (124) have led them to conclude that preparations of human plasminogen contain a proactivator, converted by streptokinase to a plasminogen activator, which in turn transforms plasminogen to plasmin. Astrup & Sterndorff (125) have reported that normal human urine contains large amounts of an activator of plasminogen and smaller amounts of a trypsin inhibitor. They report the separation of the two factors by adsorption of the activator with calcium phosphate.

Bacterial proteinases.—DeBallis *et al.* (126) have described the fractionation of the proteolytic enzymes of *Clostridium histolyticum* by means of ammonium sulfate to yield a proteinase fraction, a collagenase, an amidase-esterase, and a peptidase fraction. The proteinase is believed to be the same as the δ-enzyme described by Mandl *et al.* (127) but is about 50 times more potent in its ability to hydrolyze such substrates as azocoll, gelatin, casein, or hemoglobin. Collagenase is active against native collagen, azocoll, and gelatin, but is inactive toward casein, hemoglobin, albumin, and fibrin. The amidase-esterase is considered to be different from the collagenase; it is strongly activated by cysteine and hydrolyzes benzoylargininamide and arginine ethyl ester. The peptidase fraction is composed of several enzymes; one is a cobalt-activated dipeptidase which hydrolyzes such substrates as leucylglycine and leucylalanine; another is an aminopolypeptidase which apparently requires no activation in attacking such compounds as leucylglycylglycine and a valyl decapeptide. Mandl & Zaffuto (128) reported that peptidases occur in the filtrates of *C. histolyticum* as soon as growth of the organism begins, and therefore may be classified as extracellular enzymes. Monier *et al.* (129) have reported that a cysteine-inhibited proteinase obtained from culture filtrates of *C. histolyticum* can be distinguished from an-

other cysteine-inhibited proteinase also found in culture filtrates of this organism by their respective pH dependence curves and by their denaturation rates at 35°C. in the presence of ethylenediamine tetraacetate. However, they are both protected by Ca^{++} against heat denaturation and denaturation during dialysis at 4°C. Dresner & Schubert (130) have compared the susceptibility, to collagenase and trypsin, of collagen, soluble collagen, and renal basement membrane.

Damodaran *et al.* (131) have examined the proteolytic activity of the extracellular fluid of a strain of *Bacillus lichenoformis*. Both proteinase and peptidase activities were found. In this connection, it is of interest that *B. licheniformis* was the most frequent organism encountered in the rumen of sheep (132).

Cell-free extracts obtained by sonic disintegration of *Bacillus brevis* (ATCC 9999), a strain which hydrolyzes Gramicidin S, has been shown to attack triornithine and polyornithine (133).

Gorini & Lanzavecchia (134) have shown that the extracellular proteinase of *Coccus P* is liberated into the culture medium in the form of an inactive proenzyme and is converted to the active enzyme by an autocatalytic reaction. Under conditions where the proteinase activity is not inhibited, the appearance of the zymogen is transitory, and its presence may not be detected.

Baudet & Hagemann (135) have described the purification of penicillinase from *Bacillus cereus* by means of acetone precipitation, ammonium sulfate fractionation, and ion exchange chromatography on Amberlite IR4B. Vaczi & Uri (136) have studied an endopenicillinase from *Staphyloccocus pyogenes* and an exopenicillinase from *Bacillus subtilis*. Brown *et al.* (137) have reported that cell-free extracts of *Acetobacter suboxydans* exhibit enzymic activity toward methyl pantothenate, pantothenamide, pantothenyl-β-alanine and pantothenylglycine to yield free pantothenic acid.

Transamidation Reactions and Peptide Bond Synthesis

Williams & Thorne (138, 139) and Williams *et al.* (140) have presented further reports on the transfer reactions catalyzed by an exoenzyme from *B. subtilis*. Incubation of γ-D-glutamyl-D-glutamic acid with the enzyme system results in the formation of free glutamic acid and a compound believed to be a tripeptide of glutamic acid. This reaction occurs optimally at pH 9.0 and, at appropriate substrate concentrations, no demonstrable hydrolysis is observed; free glutamic acid and tripeptide appear at equal rates and in equimolar quantities. When hydroxylamine was present in the incubation mixture, a hydroxamic acid was formed. Although both the γ-D-glutamyl-D-glutamic acid and γ-L-glutamyl-L-glutamic acid were active as substrates, the corresponding alpha derivatives were inactive. When γ-D-glutamyl-D-glutamic acid, labeled with C^{14} in the free gamma carboxyl group, was incubated with the enzyme system, a peptide with only one labeled glutamic acid residue was obtained. The following mechanism has been suggested:

$$\gamma\text{-D-glu-D-glu}^* + \text{enzyme} \rightleftharpoons (\text{enzyme-glutamic acid}) + \text{D-glutamic acid}^*$$
$$(\text{Enzyme-glutamic acid}) + \gamma\text{-D-glu-D-glu}^* \rightleftharpoons \gamma\text{-D-glu-D-glu-D-glu}^* + \text{enzyme}$$
$$\text{Sum: } 2\ \gamma\text{-D-glu-D-glu}^* \rightleftharpoons \gamma\text{-D-glu-D-glu-D-glu}^* + \text{D-glutamic acid}^*$$

Under the conditions of the experiments of Williams *et al.* (140), the extent of the transamidation reaction between L-glutamine and D-glutamic acid, catalyzed by the *B. subtilis* preparation, exceeded the extent of hydrolysis. A study of the efficiency of the transpeptidation reaction showed that about 63 per cent of the L-glutamine that had undergone reaction participated in the transfer. Prolonged incubation of L-glutamine and D-glutamic acid with the enzyme preparation led to the production of compounds thought to be glutamic acid peptides.

The same *B. subtilis* enzyme preparation catalyzes the transfer of γ-glutamyl radicals from the polypeptide produced by this organism to either D-glutamic acid or to hydroxylamine. The hydrolysis of the polypeptide occurs maximally at pH 8.8, whereas transpeptidation and hydroxamic acid formation is optimal at pH 8.5. Thorne *et al.* (141) have investigated the various factors that influence the production of polypeptide by *B. subtilis.*

Hird & Springell (142) have described the purification of γ-glutamyl transferase from sheep kidney and its action at the γ-glutamyl bond of glutathione. The pH optimum for hydrolysis was found to be about 6, whereas at pH 7.4 to 8.5, transfer reactions occurred.

The possible relation between transpeptidation reactions and the synthesis of glutathione (GSH), glutamine, or asparagine (143) makes it desirable to mention some recent advances in this area. Snoke (144) has described the purification from yeast of an enzyme preparation that catalyzes the reaction:

$$\gamma\text{-L-Glutamyl-L-cysteine} + \text{glycine} + \text{ATP} \rightarrow \text{GSH} + \text{ADP} + P_i$$

Snoke & Bloch (145) have shown that this enzyme preparation catalyzes the replacement of the glycine portion of glutathione by C^{14}-glycine, in the presence of Mg^{++} and ADP or ATP, as well as of arsenate or phosphate. It appears that the enzyme responsible for the exchange is the same as the one which catalyzes glutathione synthesis since the ratio of synthesis to exchange remains constant during the purification. Both reactions proceed optimally at pH 8.3. Hydroxylamine can also serve as a replacement agent but under somewhat different experimental conditions than for the glycine exchange. Mandeles & Bloch (146) have described the partial purification from swine liver of an enzyme preparation that catalyzes, in the presence of ATP, the synthesis of γ-L-glutamyl-L-cysteine from the component amino acids. Webster & Varner (147) have studied the formation of glutathione from γ-glutamylcysteine and glycine by an enzyme preparation obtained from wheat germ extracts. In the presence of ATP, Mg^{++}, and K^{+}, this enzyme preparation also catalyzes the formation of a hydroxamic acid, if hydroxylamine is added in place of glycine.

Fry (148) has reported experiments on the formation of glutamine by enzyme preparations from *Micrococcus pyogenes* var. *aureus;* these also cata-

lyze the formation of glutamylhydroxamic acid from glutamic acid and hydroxylamine in the presence of ATP and Mg^{++} at pH 7.5. The glutamine-synthesizing enzyme system from peas has been shown by Staehelin & Leuthardt (149) to catalyze the exchange of ADP^{32} with ATP. Dénes (150) has examined the effect of activating ions on the stereospecificity of the glutamine synthetase reaction, catalyzed by a preparation from pigeon liver. Rudnick *et al.* (151) have examined the glutamine synthetase and glutamyl transferase activity of developing chick embryos.

The biosynthesis of asparagine has been studied in lupine seedling ex-extracts and wheat germ extracts by Webster & Varner (152). This system has been found to be similar in its general properties to the glutamine synthetase system. Meister *et al.* (153) have reported that a purified ω-amidase preparation from rat liver catalyses the hydrolysis of α-ketoglutaramic, α-ketosuccinamic, glutaramic, and succinamic acids, and also transamidation reactions of these substrates with hydroxylamine. Preparations of guinea pig serum asparaginase and *Escherichia coli* glutaminase were also found to catalyze hydroxamic acid formation from asparagine and glutamine, respectively.

Connell *et al.* (154) have described extremely valuable chromatographic techniques for the study of enzymic transpeptidation reactions. It has been suggested by Haurowitz & Horowitz (155) that enzymatic transpeptidation can be demonstrated by adding a radioactive substrate and noting its incorporation into the reaction products. By the use of this method, it has been concluded that transpeptidation occurs in plastein formation.

Use of Proteolytic Enzymes in Studies of Protein Structure and Enzyme Action

The value of purified proteolytic enzymes for the elucidation of the amino acid sequence of proteins has been clearly demonstrated by the work of Sanger on the structure of insulin. In continuation of these studies, Sanger *et al.* (156) have treated the A-chain and B-chain of oxidized insulin with a crystalline mold proteinase, and, from an examination of the resulting peptides, have determined the positions of the amide groups of insulin. The major sites of action of the mold proteinase were the bonds between the following pairs of amino acid residues: isoleucyl-valyl, glutamyl-glutaminyl, cysteyl-seryl, seryl-leucyl, leucyl-tyrosyl, tyrosyl-glutaminyl, glutaminyl-leucyl, glutamyl-asparaginyl, phenylalanyl-valyl, asparaginyl-glutaminyl, cysteyl-glycyl, histidyl-leucyl, glutamyl-alanyl, leucyl-tyrosyl, tyrosyl-leucyl, glycyl-phenylalanyl, phenylalanyl-tyrosyl, prolyl-lysyl, lysyl-alanine. The A-chain was subjected to the action of crystalline papain, and the major sites of cleavage were found to be as follows: glutamyl-glutaminyl, cysteyl-seryl, glutaminyl-leucyl, leucyl-glutamyl, glutamyl-asparginyl. The use of chymotrypsin for the cleavage of intact insulin, followed by the fractionation and identification of the resulting cystine peptides, has permitted Ryle *et al.* (157) to establish the position of the disulfide bonds of insulin.

Work in three different American laboratories on the structure of large

peptides having corticotropic activity has involved the use of specific proteolytic enzymes for the elucidation of the amino acid sequence. Bell *et al.* (158, 159) have shown that swine β-corticotropin is a single peptide chain of 39 amino acids and is cleaved by pepsin into large basic peptides and a group of small acidic peptides, with the major sites of cleavage at the following bonds: leucyl-alanyl, phenylalanyl-prolyl, aspartyl-glutaminyl. With trypsin, the major sites of cleavage were at lysyl-valyl, arginyl-tryptophyl, lysyl-lysyl, lysyl-arginyl, and arginyl-arginyl. With chymotrypsin, the major sites were tyrosyl-seryl, phenylalanyl-arginyl, tryptophyl-glycyl, leucyl-glutamyl. From the nature of the peptides obtained, the following amino acid sequence was inferred: Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys.Pro.Val.Gly. Lys.Lys.Arg.Arg.Pro.Val.Lys.Val.Tyr.Pro.Asp.Gly.Ala.Glu.Asp.Glu(NH_2). Leu.Ala.Glu.Ala.Phe.Pro.Leu.Glu.Phe.

Harris & Li (160) have described the action of carboxypeptidase on sheep α-corticotropin, and have established the C-terminal sequence -Pro. Leu.Glu. Phe. In a subsequent publication, Li *et al.* (161) have presented the results of their studies on the amino acid sequence of α-corticotropin, by the use of pepsin, trypsin and chymotrypsin, and partial acid hydrolysis of the DNP-peptide. By means of pepsin, the C-terminal sequence, -Asp.Glu.Ala.Ser.Glu. Ala.Phe.Pro.Leu.Glu.Phe. was established. In these experiments, the cleavage of an alanyl-seryl bond by pepsin was observed. The studies with trypsin gave the N-terminal sequence: Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try. Gly.Lys.Pro.Val.Gly.Lys.Lys.Arg.Arg.Pro.Val.Lys-. The remaining interior sequence was found to be: -Val.Tyr.Pro.Ala.Gly.Glu.Asp-.

White & Landmann (162) have used pepsin and trypsin to determine the amino acid sequence of swine corticotropin A, a peptide of 39 amino acids very similar to β-corticotropin. Upon treatment with pepsin, a pentapeptide was isolated, to which the structure Ser.Tyr.Ser.Met.Glu was assigned. This is the N-terminal sequence of the first five amino acids of corticotropin A. Decisive proof of the correctness of this structure was provided by the unequivocal synthesis of the pentapeptide, and the demonstration of its identity with the material obtained from the peptic digest (163). White (164) has also shown that when the purified aminopeptidase preparation of Spackman *et al.* (2) is allowed to act on corticotropin A, serine and tyrosine are released; when more than one-half of the N-terminal serine and the adjacent tyrosine had been removed, about two-thirds of the ACTH activity was lost.

It lies beyond the scope of the present review to discuss the structure of the peptides having corticotropic activity, but the brilliant work by the groups of Bell, of White, and of Li, which has led to the elucidation of the amino acid sequence of active peptides further illustrates the importance of purified proteolytic enzymes in the study of protein structure. Their findings show generally satisfactory agreement between the available knowledge of the specificity of trypsin and chymotrypsin, as determined with synthetic substrates, and the action on large peptides of complex structure. They also underline the incomplete status of our knowledge of the specificity of crystalline pepsin, as pointed out previously by several investigators.

A noteworthy recent addition to the proteinases and peptidases used in earlier work for studies of amino acid sequences is the purified aminopeptidase from swine kidney. Hill & Smith (165) have presented a preliminary report on the action of this enzyme on β-lactoglobulin, insulin, the B-chain of oxidized insulin, and mercuripapain. The use of the aminopeptidase preparation for work with corticotropin-A has already been mentioned; it may be expected that it will be as useful for the determination of N-terminal sequences as carboxypeptidase is for the study of the C-terminal sequence of peptide chains.

The knowledge of the specificity of purified proteinases has been useful in the elucidation of the structure of the posterior pituitary hormones, vasopressin, and oxytocin. The release of glycinamide from arginine-vasopressin (166) suggested the cleavage of an arginyl-glycyl bond, whose presence was unequivocally established in subsequent studies. The presence of glutamine and asparagine residues in oxytocin and vasopressin was established by treatment with H_2S-activated papain (167): earlier work (168) had shown that papain does not hydrolyze the γ-amide bond of glutaminyl derivatives.

Studies in the laboratory of Linderstrøm-Lang on the enzymic breakdown of ribonuclease have involved the use of crystalline subtilisin, which causes an extensive degradation of the substrate (169). However, if the action of subtilisin is not prolonged, a modified fully active derivative of ribonuclease is formed (169, 170, 171). Anfinsen (172) has extended his earlier study (173) of the action of pepsin on ribonuclease, which is accompanied by a very rapid loss of ribonuclease activity. Kalnitsky & Anderson (174) have reported that carboxypeptidase releases the C-terminal amino acids of ribonuclease in the order to be expected from earlier studies (175), and that there was no decrease in ribonuclease activity during the period of carboxypeptidase action. Although it is not possible at the present writing to define completely the sequence of the 126 amino acid residues and the position of the disulfide bonds of ribonuclease, it seems likely that this information will soon become available and that it will be possible to interpret more clearly the effect of various proteinases on this enzyme.

Some years ago, Tsou (176) described a heme peptide obtained by the digestion of horse cytochrome-*c* by crystalline pepsin. Tuppy & Paléus (177) have prepared the conjugated peptide from beef cytochrome-*c*, have purified it by partition chromatography, and have shown the peptide moiety to be composed of eleven amino acid residues arranged in the following sequence: Val.Glu(NH_2).Lys.CySH.Ala.Glu(NH_2).CySH.His.Thr.Val.Glu. The two cysteine residues are linked by thioether bonds to the side chains at positions 2 and 4 of the porphyrin ring, as suggested many years ago by Theorell for intact cytochrome-*c*. The peptide component of pepsin-treated salmon cytochrome-*c* also had the above sequence, whereas in the material from chicken cytochrome-*c*, the alanyl residue was replaced by a seryl residue. The action of trypsin on beef cytochrome-*c* leads to the formation of heme peptide with the sequence CySH.Ala.Glu(NH_2).CySH.His.Thr.Val.Glu.Lys (177, 178). The treatment of the porphyrin-free peptide (from the pepsin-treated cyto-

chrome-*c*) with subtilisin gave a number of dipeptides (177). Leaf & Gillies (179) have largely confirmed the findings of Tuppy & Paléus, but report that after removal of the prosthetic group at least two peptide fractions could be separated.

The work of Harris & Knight (180) has shown that the action of carboxypeptidase on tobacco mosaic virus (TMV) leads to the rapid release of about 2,900 residues of threonine as the sole amino acid per mole of TMV, assuming a molecular weight of 50,000,000. It has been concluded that the threonine residues represent the C-terminal groups of 2900 equal subunits of molecular weight *ca.* 17,000. The carboxypeptidase-treated TMV, like the untreated virus, is resistant to the action of trypsin or chymotrypsin. Knight (181) has found that, for each of 13 strains of TMV, carboxypeptidase action led to the release of only threonine, whereas with other plant viruses (potato X, southern bean mosaic, tomato bushy stunt, tobacco ring-spot, and cucumber 3 and 4), smaller amounts of several other amino acids were released by carboxypeptidase. It would appear, therefore, that carboxypeptidase action on TMV stops after the rapid release of C-terminal threonine; with other viruses, the enzyme successively cleaves peptide bonds along the chains, or the chains of a given virus differ in their C-terminal groups.

Earlier work of Cori & Cori (182) had shown that the restricted action of crystalline trypsin on phosphorylase-*a* simulated the action of an enzyme (from rabbit muscle) named the PR-enzyme. Subsequent work showed that the action of PR-enzyme on phosphorylase-*a* is associated with the halving of the molecule to form inactive phosphorylase-*b*; the PR-enzyme was therefore renamed "phosphorylase-rupturing" enzyme. More recent studies by Keller & Cori (183) have led to the purification of the PR-enzyme, and have permitted a comparison of its action on phosphorylase-*a* with that of crystalline trypsin (184). The phosphorylase-*b* preparations formed by trypsin and by the PR-enzyme have the same sedimentation constants, but somewhat different electrophoretic behavior. The trypsin-catalyzed conversion of phosphorylase-*a*, like the action of the PR-enzyme, is inhibited by AMP. An examination of the effect of the purified PR-enzyme on benzoyl-L-arginine ethyl ester, L-arginine ethyl ester, and L-lysine ethyl ester showed no hydrolysis under the conditions used; however, L-arginine ethyl ester and L-lysine ethyl ester were found to be competitive inhibitors of the action of the PR-enzyme on phosphorylase-*a* (185). The PR-enzyme had no demonstrable effect on myosin or aldolase, and did not activate chymotrypsinogen; it must be concluded therefore, that if the PR-enzyme is an endopeptidase, its specificity is different from that of crystalline trypsin.

Gergely *et al.* (186, 187) have shown that the action of chymotrypsin on myosin is similar to that reported earlier for trypsin. There is a rapid decrease in the viscosity of myosin, and two fractions may be isolated from the digest; the more rapidly sedimenting fraction carries the adenosinetriphosphatase activity. Light scattering studies (187) on the pairs of fractions obtained by treatment of myosin with trypsin or chymotrypsin, have shown that the molecular weight of the two adenosinetriphosphatase fractions is about

500,000, and of the enzymatically inactive fractions is 500,000 to 700,000. These results appear to be inconsistent with previous conclusions drawn from sedimentation and diffusion studies.

Courts (188) has studied the action of several proteinases on gelatin, and has used the fluorodinitrobenzene method to determine the nature of the N-terminal residues that are released. The action of pepsin is accompanied principally by the release of N-terminal alanyl and valyl residues; with trypsin, glycyl residues; with papain, alanyl residues. In addition, these three enzymes, and chymotrypsin, exhibit a "secondary specificity" that involves the release of seryl, threonyl, aspartyl, glutamyl, alanyl, and glycyl residues.

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CHEMISTRY OF THE CARBOHYDRATES[1]

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The flow of publications concerned with carbohydrates has continued unabated so that it has been necessary to restrict this review to selected topics.

Sulphur-Containing Carbohydrate Derivatives

Sugar mercaptals and thioglycosides.—A growing interest is being shown in the reactions of sugar mercaptals with oxidizing agents. In 1950 Huebner, Pankratz & Link (1) reported that the diethyl mercaptals of certain fully substituted *aldehydo*-sugars, when treated with lead tetra-acetate in glacial acetic acid, consumed one molar proportion of the oxidant rapidly and then a second more slowly. Baker (2) found that considerably more lead tetra-acetate than this was ultimately consumed by dibenzyl mercaptal groups in sugars. More recently (3), the primary products of the reaction were identified as the dialkyl disulphide (RSSR) and the diacetate of the substituted *aldehydo*-sugar [$X \cdot CH(OAc)_2$]; there is a complication, however, inasmuch as the dialkyl disulphide subsequently reacts with more lead tetra-acetate. A possible mechanism for the initial cleavage, based on ionization of the parent mercaptal, is as follows (3):

$$X \cdot CH(SR)_2 + HOAc \rightleftharpoons X \cdot C^{+}H(SR) + (SR^{-}) + (OAc^{-}) + H^{+} \rightleftharpoons X \cdot CH(SR)(OAc) + RSH$$
$$X \cdot CH(SR)(OAc) + HOAc \rightleftharpoons X \cdot C^{+}H(OAc) + (SR^{-}) + (OAc^{-}) + H^{+} \rightleftharpoons X \cdot CH(OAc)_2 + RSH$$
$$2RSH + Pb(OAc)_4 \rightarrow RSSR + Pb(OAc)_2 + 2HOAc$$
$$\text{Over-all reaction: } X \cdot CH(SR)_2 + Pb(OAc)_4 \rightarrow RSSR + X \cdot CH(OAc)_2 + Pb(OAc)_2$$

In conformity with this mechanism, toluene-ω-thiol yields dibenzyl disulphide when treated with lead tetra-acetate in glacial acetic acid, or in benzene-acetic acid. The rate of consumption of the oxidant by glucose dibenzyl mercaptal penta-acetate in acetic acid is increased by the introduction of water and decreased by benzene, presumably as a result of the influence of these solvents on the ionization step (3). This pronounced retardation of the reaction by benzene (and by other nonionizing solvents) is not observed during the cleavage of glycols by lead tetra-acetate. It is for this reason that Schmidt & Wernicke (4) and Wolfrom & Usdin (5), using nonionizing media, were able to oxidize glycol groups in sugar mercaptals without affecting the sulphur moieties. Likewise, an explanation is afforded of Baker's observation (2) that the glycol group of 4,5-O-isopropylidene-D-

[1] The survey of the literature pertaining to this review was concluded in September, 1955.

[2] The authors are indebted to Professor M. Stacey, F.R.S. and Dr. A. B. Foster for helpful advice.

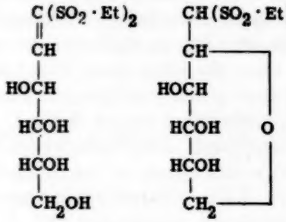
arabinose dibenzyl mercaptal was attacked more readily by lead tetra-acetate than was the mercaptal group, whereas the order was reversed in the case of 2,3-di-O-benzoyl-D-arabinose dibenzyl mercaptal, for the former reaction was conducted in benzene and the latter in acetic acid.

Other reagents which will remove a thiol from the above equilibria will, under ionizing conditions, also bring about demercaptalation; examples are bromine, iodine, and hydrogen peroxide in acetic acid, and mercuric chloride, periodate, and acidified iodate in water (3). In some of these cases, however, carbohydrate sulphoxides and sulphones are produced in competing reactions. The extent to which each of the simultaneous reactions proceeds with a given oxidant is determined, *inter alia*, by the ionizing power of the medium and by the dissociation constant of the mercaptal; thus whereas the very stable formaldehyde dibenzyl mercaptal gives almost exclusively the disulphoxide with hydrogen peroxide in acetone, benzaldehyde dibenzyl mercaptal yields principally dibenzyl disulphide under the same conditions (6). Zinner & Falk (7) reported the conversion of sugar mercaptals into the corresponding disulphoxides [$X \cdot CH(SO \cdot R)_2$] with hydrogen peroxide in acetic acid but gave no evidence to eliminate the possibility that the products were the isomeric mono-sulphones [$X \cdot CH(SO_2 \cdot R)(SR)$]. However, their claim is strengthened by the fact that the product from the analogous oxidation of formaldehyde dibenzyl mercaptal shows strong infrared absorption bands characteristic of the sulphoxide group (6). It is interesting that this should be so since hydrogen peroxide converts disulphides into thiolsulphonates ($R \cdot SO_2 \cdot SR$) (8). Zinner & Falk (7) found that, with hydrogen peroxide containing ammonium molybdate, sugar mercaptals were oxidized beyond the disulphoxide stage to give disulphones, similar to those reported earlier by MacDonald & Fischer (9).

Oxidations of sugar mercaptals with permanganate in aqueous acetic acid are even more complex; the principal reaction in the case of D-glucose dibenzylmercaptal penta-acetate appears to be the formation of a mixture of the diastereoisomeric monosulphones, but these are apparently accompanied by the corresponding disulphoxide and by one of the D-*arabo*-3,4,5,6-tetra-acetoxy-1-benzylsulphonyl-1-benzylthio-hex-1-enes, formed from the main components by loss of the elements of acetic acid (6). [It is significant that Bonner & Drisko (10) could not obtain sulphoxides from thioglucosides using permanganate, whereas Micheel & Schmitz (11) had prepared them in good yield using hydrogen peroxide.] Treatment of the main product with ammonia in methanol gave a 2-acetamido-1-benzylsulphonyl-1-benzylthio-3,4,5,6-tetrahydroxyhexane for which four structures are possible, two in the D-glucose series and two with the D-mannose configuration. Unfortunately an attempt to characterize this material by using an alternative preparation based on penta-acetyl-D-glucosamine dibenzylmercaptal was handicapped by difficulty encountered in preparing the mercaptal (cf. 12, 13). In an analogous reaction, MacDonald & Fischer (9) converted D-*arabo*-3,4,5,6-tetra-acetoxy-1,1-diethylsulphonylhex-1-ene, by treatment with

methanolic ammonia, followed by acetylation, into D-*gluco*-2-acetamido-3,4,5,6-tetra-acetoxy-1,1-diethylsulphonylhexane.

The stereochemical and structural complexities encountered with partially oxidized mercaptals are greatly reduced in the case of the disulphones and this, together with the excellent methods of preparation available which involve peracid oxidations of either substituted or unsubstituted sugar mercaptals (7, 9, 14 to 19), has enabled more rapid progress to be made with these compounds. The aldose-derived disulphones [$X \cdot CH(OH) \cdot CH(SO_2 \cdot R)_2$] readily lose the elements of water (and their acetates eliminate acetic acid) so that the compounds isolated usually carry an olefinic group [$X \cdot CH{:}C(SO_2 \cdot R)_2$]; the elimination cannot occur with ketose-derived disulphones. However, both aldose and ketose types undergo a reversed Knoevenagel reaction in the presence of bases, e.g., in the aldose case, $X \cdot CH{:}C(SO_2 \cdot R)_2 \rightarrow X \cdot CH(OH) \cdot CH(SO_2 \cdot R)_2 \rightarrow X \cdot CHO + CH_2(SO_2 \cdot R)_2$, and thus provide new routes for descent of the sugar series (9, 14 to 20). Some transformations reported are: aldohexoses into aldopentoses (9, 14, 16, 17), fructose into erythrose derivatives (18, 19) and myoinosose-2 into *xylo*-trihydroxyglutardialdehyde (15, 18). Like water and ammonia (see above), methanol will add to the olefinic disulphones; for example, D-*arabo*-3,4,5,6-tetra-acetoxy-1,1-diethylsulphonylhex-1-ene yields a 2-methyl ether which may have either the D-glucose or the D-mannose configuration (19). This problem could be resolved by isolation of the oxidation product from 2-O-methyl-D-glucose diethyl mercaptal tetra-acetate, which has become more readily available through Lemieux & Bauer's discovery (21) that acetyl migrations in D-glucose diethylmercaptal tetra-acetate permit either 2- or 4-derivatives to be prepared. By analogy with the addition reactions described, it was suggested (19) that an aldohexose-derived disulphone (I) may exist, under suitable conditions, in equilibrium with the cyclic form (II), and this was in fact proved in simultaneous independent studies (20); the aldopentose-derived disulphones cannot assume a six-membered ring structure and appear to be acyclic (20).



I II

Several new substituted sugar mercaptals have been prepared. Thus Bolliger & Schmid (22) have described the properties of 4,5,6-tri-O-benzoyl-

D-galactose diethyl mercaptal and the conversion of 2,3,4,5-tetra-O-benzoyl-6-O-trityl-D-glucose diethylmercaptal into the 3,4,5,6-tetrabenzoate and the 2-thioethyl ether thereof. Barclay, Foster & Overend (23), during syntheses of phosphates of *aldehydo*-sugars, have prepared a number of derivatives of D-glucose and D-galactose diethyl mercaptals carrying acetyl, benzoyl, methanesulphonyl, triphenylmethyl, diphenylphosphoryl, and deoxy groups. 6-Deoxy-6-thioethyl-D-galactose and its diethyl mercaptal have been synthesized by two routes which defined their structures (24). Mercaptolyses of agar, carrageenin, and the polysaccharide from *Chondrus crispus* have yielded *inter alia*, 3,6-anhydrogalactose diethyl mercaptal (25, 26). Because of the high resistance of acetylated ethyl thioglycosides to anomerization, Lemieux & Brice (27) were able to use the method of mercaptolysis with advantage in a study of the participation of neighboring groups in replacements of acetoxy-groups at C-1 of sugars.

Dithiols and thioethers.—Harding & Owen (28) have explained abnormalities encountered during saponifications of the acetates of 2′,3′-dimercaptopropyl β-D-glucoside and 2′3′-dimercaptopropyl ethers of polyols by quantitative studies of the alkaline hydrolysis of their simple analogues. Subsequently, 3′,4′-dimercaptobutyl β-D-glucoside and D-mannitol-1- and D-glucitol-6-(3′,4′-dimercaptobutyl) ethers were synthesized as their acetates, which were partially converted to cyclic sulphides by barium methoxide (29). Similarly, there was some cyclization during the deacetylation stage in the preparation of the O-β-D-glucoside of 1,3-dimercaptopropan-2-ol (29). Treatment of dibromopropanols (and their acetates) with the sodio-derivative of toluene-ω-thiol has yielded S-benzyl derivatives of dithioglycerols and thence the dithioglycerols themselves (30, 31). A synthesis of 2′,3′-bisbenzylthiopropyl 2,3,4,6-tetra-O-acetyl-β-D-glucoside has been described (30).

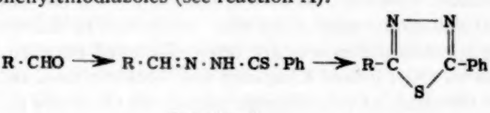
Sulphonate esters.—Since Tipson's review (32) of sulphonate esters in 1953, interest has continued in these esters as possible precursors of deoxy-sugars. With lithium aluminium hydride, the 2-toluene-*p*-sulphonate and the 2-methanesulphonate of methyl 3,4-O-isopropylidene-β-L-arabinoside each gave methyl 3,4-O-isopropylidene-β-L-arabinoside (33), and methyl 3-O-methyl-2,4,6-tri-O-toluene-*p*-sulphonyl-β-D-idoside yielded methyl 6-deoxy-3-O-methyl β-D-idoside (34); in the latter case the primary ester was attacked more rapidly than the other two. These results agreed with an earlier observation (35) that primary sulphonate esters of aldoses are converted into deoxy-compounds (alkyl-oxygen fission), whereas secondary esters usually give the parent sugar (sulphonyl-oxygen fission). Subsequently, lithium aluminium hydride was shown to convert both the 5- and the 3,5-toluene-*p*-sulphonates of 1,2-O-isopropylidene-L-arabinose into 5-deoxy-1,2-O-isopropylidene-L-arabinose, as expected (36). However, complications have arisen during the action of lithium aluminium hydride on certain secondary sulphonates carrying free hydroxyl groups. Thus, (*a*) methyl 2-O-methanesulphonyl (or 2-O-toluene-*p*-sulphonyl) β-L-arabinoside gave a mixture of methyl-β-L-arabinoside, 2,3-anhydro β-L-riboside, 2-deoxy-β-L-riboside, and 3-deoxy-β-L-xyloside (33), (*b*) methyl 4,6-O-benzylidene-2,3-di-

O-toluene-*p*-sulphonyl-α-D-glucoside yielded methyl 4,6-O-benzylidene-3-deoxy-α-D-glucoside (37), and (*c*) 1,3-4,6-di-O-methylene-2,5-di-O-toluene-*p*-sulphonyl-D-mannitol gave 1,3-4,6-di-O-methylene-D-mannitol together with a 2,5-anhydro-derivative (36). The conclusion is inescapable that, in appropriate cases, direct hydrogenolysis of the secondary sulphonate to the alcohol is accompanied by the formation, and subsequent rupture, of an anhydro-ring, to yield deoxy-compounds (33, 36, 37). L-Idose derivatives have been prepared from D-glucose during a study of the replacement of tosyloxy-groups by acetoxy-groups (38). The reaction between O-hydroxyethyl cellulose and toluene-*p*-sulphonic acid has been examined (39).

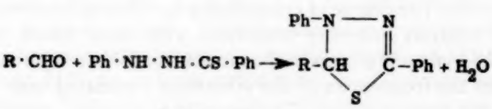
The failure of certain derivatives of the 2- and 3-toluene-*p*-sulphonates of 1,6-anhydro-β-D-altrose to react with sodium methoxide has been attributed to steric hindrance (to the formation of dianhydrohexoses) by the axial groups at C-1, C-4, and C-5 in the 1C conformation (40). Sulphonates, sulphinates and nitrates of methyl 4,6-O-benzylidene-α-D-glucoside have been prepared by Honeyman & Morgan (41), who found that sodium iodide in acetone at 100°C. for 20 hr. was ineffective with the ditoluene-*p*-sulphonate and the dimethanesulphonate, in contrast to the dinitrate. A study of Walden inversions in sulphonates of glucosamine derivatives has been reported by Jeanloz (42).

Miscellaneous sulphur compounds.—Foster & Wolfrom (43) have shown that, whereas pyrolysis of the xanthate esters of hydroxy-compounds often results in olefin formation (Chugaev reaction), it gives no detectable olefins from the 2-(S-methylxanthate) and the 2-(S-triphenylmethylxanthate) of methyl 3,4-O-isopropylidene-β-D-arabinopyranoside or the 2-(S-methylxanthate) of methyl 4,6-O-benzylidene-3-O-methyl-α-D-altropyranoside. A pyrolysis product from the first-named xanthate yields methyl 2-deoxy-3,4-O-isopropylidene-β-D-riboside when subjected to reductive desulphurization. This method may well provide a new general route to deoxy-sugars; it is believed to involve the rearrangement R·O·CS·SR′→RS·CO·SR′ (43).

Various reactions of carbohydrates with compounds containing both nitrogen and sulphur have been reported. Thus Micheel & Brunkhorst (44) have synthesized glycosyl derivatives of thioureas, and Holmberg (45) has prepared hexuronic acid thiobenzhydrazones and oxidized them with ferric chloride to phenylthiodiazoles (see reaction A).



Reaction A



Reaction B

Holmberg (46) subsequently synthesized L-fucose thiobenzhydrazone and studied its reactions with ferric chloride, DL-α-bromopropionic acid, and formaldehyde. In a related series of reactions (47) he prepared glycothiodiazolines from various aldoses by treatment with α-phenyl-β-thiobenzoylhydrazine (i.e., 2-phenyl-thiobenzhydrazide) in alcoholic hydrogen chloride; for a general representation see reaction B.

Since the glycothiodiazolines are formed quite readily and have very high specific rotations, they may constitute a useful basis for a new analytical method (47). A serious disadvantage, however, is that they are produced as mixtures of stereoisomers which have not yet been separated satisfactorily. In a similar fashion, Westphal and co-workers (48) have characterized sugars by their reaction with sulphonylhydrazides.

Infrared Spectroscopy of Carbohydrates

Infrared spectroscopy has already proved extremely useful in carbohydrate chemistry and is likely to be employed far more widely in the future. Its main applications have been concerned so far with such problems as the detection of substituents which display characteristic absorption bands and the identification of unknown carbohydrate samples by direct comparisons of their spectra with those of authentic specimens. However, during the last few years, part of the task of assigning observed bands to particular vibrational modes in carbohydrate molecules has been accomplished. Although much still remains to be done, it is clear that it will be possible in the future to derive much information about the structure, configuration, and conformation of a new carbohydrate merely from an examination of its infrared spectrum. Having rapidly gained such information, the task of obtaining confirmatory evidence by the more conventional methods should be greatly facilitated.

Kuhn (49), Fletcher & Diehl (50), and Stevenson & Levine (51) have all used infrared spectroscopy to demonstrate the identity of two carbohydrate samples or to distinguish between closely related carbohydrates. Provided that a wide frequency range is covered, comparisons of this type are likely to be at least as reliable as those based on melting points and may well be superior if one of the samples is slightly impure or if melting is accompanied by decomposition. However, infrared spectroscopy will not differentiate between optical enantiomorphs unless they crystallize in different habits (cf. 52). Striking spectral differences are often displayed between polymorphic crystalline forms (53); indeed Chapman (54) recently used such differences as a basis for the study of polymorphic transitions of certain glycerides. Passage from the crystalline to the amorphous state causes broadening of the absorption bands, with loss of definition, and possibly also slight movement of the peaks (55). The degree of crystallinity in cellulose has been determined by infrared analysis following treatment with D_2O, which preferentially deuterates the hydroxyl groups in the more accessible amorphous regions and thereby alters the frequencies of the vibrations associated with these groups (56).

Techniques.—It is desirable that compounds should be in the same physical state when their spectra are to be compared. With crystalline substances, crystal orientation is sometimes important (57). Good spectra can usually be obtained from solutions, but, with unsubstituted sugars and polysaccharides, it is difficult to find a solvent with suitable infrared transparency which will not also be detrimental to the sodium chloride cells. Consequently, solids are often finely ground in liquid paraffin ("Nujol") or in a halogenated hydrocarbon; a useful vibrating grinder has been introduced for this purpose (58). Polysaccharides should be freeze-dried from aqueous solution, finely ground in ether and then suspended in Nujol (59). Alternatively, a solution of the sample can be evaporated to dryness on a silver chloride window (49).

A noteworthy recent development in the technique of preparing solid samples for analysis is the introduction of the potassium bromide pressed plate method (60 to 68). In this method the sample is ground with dry powdered potassium bromide and the mixture is pressed (*ca.* 35 tons/sq. inch) to form a translucent pellet, which gives a spectrum free from interfering bands attributable to solvents or to mulling agents. Several techniques for the production and use of the pellets have been described (60, 62, 65, 67, 69). An attractive feature of the method is that it can be used for quantitative studies, provided that the same distribution of particle size is maintained in all samples and standards (63, 67). Ingebrigtson & Smith (67) have reported that the pellets give spectra superior to those from mineral oil mulls [a finding not in complete agreement with those of Elsey & Haszeldine (64)], but inferior to those from solutions. The quality is improved, however, if the sample is ground in the presence of a volatile solvent, grinding being continued until evaporation of the solvent is complete (67). A warning that the method may sometimes be misleading was given (66) when it was found that four out of eleven carbohydrates examined showed progressive changes in spectra during storage of the pellets. The changes could not be correlated with the presence or absence of hydroxyl-groups, as has since been done (68) with phenols, carboxylic acids, and simple alcohols. It has been suggested (68) that, in the latter case, adsorption of the sample on the alkali halide particles is responsible for such phenomena.

Group frequencies.—The use of infrared analysis for the identification of certain functional groups in carbohydrates is made possible by the fact that the atoms in these groups vibrate virtually independently of the rest of the molecule, except when influenced by such factors as conjugation or resonance with other vibrations of the same frequency (53, 70). Analyses based on group frequencies are fairly numerous in the carbohydrate field; they have been particularly valuable in investigations of the more complex structures, such as polysaccharides (49). Thus Orr (71) found the spectrum of hyaluronic acid to be consistent with its formulation as a polymer of glucuronosyl-N-acetylglucosamine; it displayed absorption bands at 1736 (carboxylic acid), 1648, and 1560 $cm.^{-1}$ (monosubstituted amide), when cast as a film from acid solution. The ratio of hexuronic acid to hexosamine was determined

from the relative intensities of the bands at 1736 and 1560 cm.$^{-1}$ In a similar study (71) two isomers of chondroitin sulphuric acid were detected; their spectra were related to that of hyaluronic acid, but each displayed an additional intense band near 1240 cm.$^{-1}$, as a result of S=O stretching in the sulphate group. By analogy with earlier work on steroids (72), Orr concluded that the sulphate group was axial in one of the isomers and equatorial in the other. The pronounced absorptions at about 1240 cm.$^{-1}$ in the spectra of κ and λ carrageenins have also been assigned to S=O stretching vibrations, and evidence has been presented that all the sulphate groups in carrageenin are equatorial (73). Levine, Stevenson & Kabler (74) have shown that the serologically-active and type specific pneumococcal polysaccharides can be distinguished from one another by infrared analysis. The samples examined were divided into four classes depending on whether their spectra exhibited absorption peaks characteristic of the amide group, the carboxyl group, both, or neither; in addition, a clear distinction could be made between O-acetyl and N-acetyl groups.

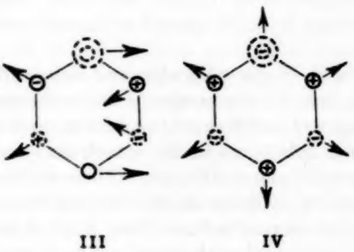
Other studies of this type have been carried out on chitin (75), dextran (76), and cellulose derivatives (77, 78). The problems surrounding the interpretation of the infrared spectra of such large molecules have been discussed by Sutherland (79). A comprehensive review of the frequencies associated with the functional groups commonly encountered in carbohydrate chemistry has been prepared recently by Barker, Bourne & Whiffen (80). It is inevitable that two or more groups should sometimes absorb at the same frequency, and it is then necessary to examine other regions of the infrared spectrum in order to decide between them. For example, the C=O stretching of acetamido groups (1648 cm.$^{-1}$) could be confused with absorptions (1645 $\pm$ 5 cm.$^{-1}$) attributable to physically-bound water (74, 81) or to water of crystallization (74, 82), but the N—H deformation mode (1560 to 1508 cm.$^{-1}$) will differentiate between these possibilities. Fauvarque (83) has used infrared spectroscopy to study the spatial dispositions of the water and carbohydrate molecules in the crystalline monohydrate of calcium gluconate.

Assignment of vibrations in carbohydrates.—Interest has developed recently in the assignment of vibrations associated with the carbohydrates themselves, rather than with substituents. The frequency range studied in greatest detail is that between 1000 and 700 cm.$^{-1}$, which falls in the "fingerprint region" (84). Attention was drawn to this range by Kuhn's observation (49) that anomeric sugars show spectral differences there and by Burket and Melvin's report (76) that certain dextrans (now known to possess 1,3 branch points) display absorption at 794 cm.$^{-1}$, whereas others do not. Steele & Pacsu (85) had already foreseen that the region from 950 to 400 cm.$^{-1}$ might prove to be the most interesting for further infrared study on polysaccharides.

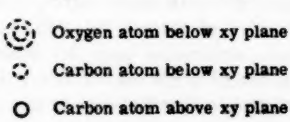
The most striking feature which emerged from an examination of the spectra of a large number of D-glucopyranose derivatives was that an absorption peak (type 2*a*) at 844 $\pm$ 8 cm.$^{-1}$, of moderate or strong intensity, was

shown by the α-anomers, while the β-anomers displayed absorption (type 2*b*) at 891 ± 7 cm.$^{-1}$ instead (59, 86). The distinction was apparent with mono-, oligo-, and polysaccharides. Those oligosaccharides in which the anomeric character of the reducing group differed from that of the glucosidic linkage (e.g., β-maltose) gave both 2*a* and 2*b* absorptions. Since the type 2 bands were related to the configuration at C-1, and since they were independent of the nature of the glycosidic substituent, they were assigned to C-1—H deformation modes, for which the observed frequencies were shown to be reasonable. Thus the 2*a* and 2*b* bands were believed to arise when the hydrogen atom at C-1 occupied the equatorial and axial positions, respectively, in the glucopyranose ring. It is apparent that C—H deformation modes involving C-1 differ in frequency from those involving other carbon atoms, probably because C-1 is the only carbon atom in pyranosides which is attached to two oxygen atoms.

The α- and β-glucopyranosides each displayed two other sets of bands (1 and 3) in the region under examination (59, 86); the frequencies were: type 1, 917 ± 13 (α-anomers) and 920 ± 5 cm.$^{-1}$ (β-anomers); type 3, 766 ± 10 (α-anomers), and 774 ± 9 cm.$^{-1}$ (β-anomers). These absorptions were, in general, less intense with β-glucosides than with α-glucosides, but otherwise they showed little or no dependence on the configuration at C-1. On the basis of Burket & Badger's (87) excellent detailed study of the infrared and Raman spectra of tetrahydropyran and *p*-dioxane, the type 1 and 3 bands were assigned (59) to ring vibrations, essentially as pictured in III and IV, respectively; in tetrahydropyran, the former vibration gives absorption at 875 cm.$^{-1}$ and the latter at 818 cm.$^{-1}$ (87). Unfortunately, some of the type 1 absorptions of the α-compounds lie in the same range as the type 2*b* absorptions of the β-compounds, so that a band at *ca.* 900 cm.$^{-1}$ is not in itself convincing evidence of the presence of a β-glucopyranose unit. The frequencies of the bands of types 1 and 3 shown by an unknown α-polyglucosan give an indication of the points of attachment of the principal glucosidic



III IV



linkages; the respective values are 930±4 and 758±2 cm.$^{-1}$ for α-1,4-polyglucosans and 917±2 and 768±1 cm.$^{-1}$ for α-1,6-polyglucosans (59, 86), while α-1,3-linkages give a band at 793±3 cm.$^{-1}$ (59, 76, 86). However, with β-polyglucosans bands 1 and 3 are either weak or absent.

Various substituted glucopyranoses, including acetates and methyl ethers, have been shown to give bands of types 1, 2*a* (or 2*b*), and 3 near the frequencies mentioned above; a complication here is that bands 1 and 2*b* may be hidden among the C—O—C vibrations of the substituents (55). Furthermore, vibrations of phenyl (88) and sulphate (71) groups may overlap bands 2*a* and 3 (55, 80).

Similar studies (55) in the galactopyranose and mannopyranose series were less conclusive because of the smaller number of compounds available; in addition, a high proportion of them were acetates, so that type 1 bands were obscured. The present indications are that bands 2*a*, 2*b*, and 3 occur, respectively, at 825±11, 895±9, and 752±20 cm.$^{-1}$ with galactose derivatives and at 833±8, 893±6, and 791±18 cm.$^{-1}$ with mannose derivatives. Further confirmation is awaited. The presence or absence of absorption between 830 and 855 cm.$^{-1}$ again offers a method of distinguishing between the α- and β-forms of arabopyranose derivatives; it is of interest that, in this series, it is the β-anomers which display the 2*a* band, as would be expected if the earlier assignment of this band to a C-1—H (equatorial) deformation mode is correct (55, 80). The xylopyranose series is exceptional among those studied to date in that neither the α- nor the β-anomers display absorption between 830 and 855 cm.$^{-1}$; fortunately, the type 3 absorption (at 749±10 cm.$^{-1}$ with α-anomers; absent with β-anomers) can be used instead to indicate the configuration at C-1 (55). The absence of type 3 absorption from the spectra of derivatives of β-xylopyranose is probably a consequence of there being no axial substituents (other than hydrogen) in these compounds, which therefore approach the centrosymmetrical state exemplified by *scyllo*inositol, for which an infrared active ring-breathing frequency is forbidden by the selection rules (55, 89). Recently Takahashi (90) has also described a study of the infrared spectra of sugars over the region 1000 to 700 cm.$^{-1}$.

In 1953, Whistler & House (91) observed independently of the above studies that the α-forms of hexoses often exhibit characteristic absorptions in the frequency range 839 to 818 cm.$^{-1}$ In addition, they noted that anomeric pairs sometimes show differences in the wavelength and intensity of their absorptions at frequencies above 1000 cm.$^{-1}$ These regions could thus furnish very useful confirmatory evidence in the determination of anomeric character. Some of the above results have been applied to studies of specific structural problems concerned with transfructosylation (92) and with the acid-catalyzed polymerization of glucose (93, 94).

A further characteristic absorption (type 2*c*), which is displayed by derivatives of mannopyranose (876±9 cm.$^{-1}$) and galactopyranose (871±7 cm.$^{-1}$), but not by the corresponding derivatives of glucopyranose, has been

assigned to C—H (equatorial) deformations involving C-2 and C-4, respectively (55); cyclitols which carry one or more equatorial hydrogen atoms show a similar band (873 ± 11 cm.$^{-1}$) (89).

Individual comparisons of spectra between quercitols and the corresponding inositols, and between deoxy-sugars and their parent sugars, have revealed that all of the deoxy-compounds give an additional band at *ca.* 860 cm.$^{-1}$ (89); this has been assigned to a CH_2 rocking vibration which Burket & Badger (87) reported at 856 cm.$^{-1}$ in the spectrum of tetrahydropyran. Likewise, absorptions at *ca.* 963 cm.$^{-1}$ exhibited by cyclitols carrying C-methyl groups (89) agree with the value given by Sheppard & Simpson (95) for rocking of an isolated C-methyl group attached to a heavy framework.

Compounds containing a furanose or hydrofuranol ring have been shown to exhibit absorption bands, designated A, B, C, and D, at 924 ± 13, 879 ± 7, 858 ± 7, and 799 ± 17 cm.$^{-1}$, respectively (96). Since the ring in tetrahydrofuran is so slightly puckered that C—H bonds projecting above and below its plane are virtually equivalent (97), differentiation between α- and β-furanosides was not expected. Band A was assigned to the ring-breathing frequency, and bands B and C to skeletal stretching in side chains and to rocking vibrations of methylene groups (96).

Mizushima & Shimanouchi (98) have determined the configurations of various high polymers, including amylose, from considerations based, in part, on their infrared and Raman spectra. A general mathematical expression was derived which describes the configuration of the chain molecule in terms of bond lengths, bond angles, and azimuthal angles of internal rotation.

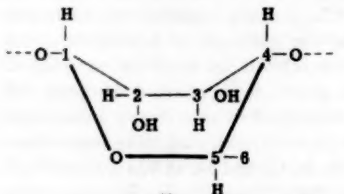
A detailed review of applications of infrared spectroscopy in carbohydrate chemistry has been prepared (80) in collaboration with our colleagues, Drs. S. A. Barker and D. H. Whiffen, to whom we make grateful acknowledgement.

Conformational Analysis of Carbohydrates

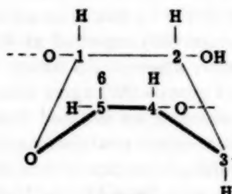
The concept of conformational analysis has brought a fresh approach to many of the problems of carbohydrate chemistry. There are several useful reviews (99 to 106) covering the literature up to 1953. The application of conformational analysis to the interpretation of infrared spectra of carbohydrates was considered earlier in this review.

In continuation of his studies of cuprammonium complexes of carbohydrates, Reeves (107) found that the glucose residues in amylose probably exist in more than one ring conformation; he expressed the view that the principal conformations may well be the unusual boat forms B1 and 3B (V and VI, respectively), but preferred not to exclude the possibility that another pair of conformations, of which one is reactive towards cuprammonium and the other unreactive, may be involved. He observed that the solubility of amylose in cuprammonium is very limited but is increased by the addition of small amounts of sodium hydroxide. Moreover, neutral amylose

solutions give precipitates with cuprammonium, in contrast with alkaline solutions; alkaline solutions of amylose which have been neutralized will then precipitate with cuprammonium. Reeves further noted that these facts agreed with the conclusion of Senti & Witnauer (108) that the glucose residues in alkali-treated amylose all possess the same conformation; he thought



V



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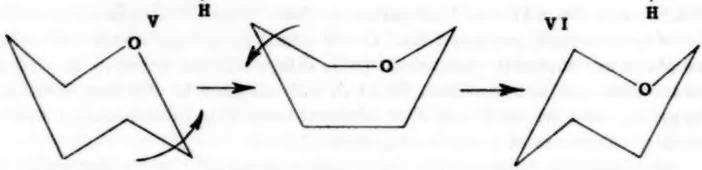


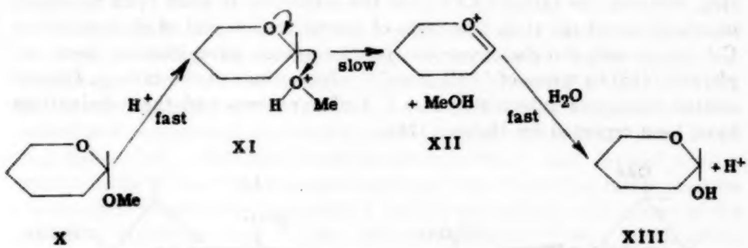
VII VIII IX

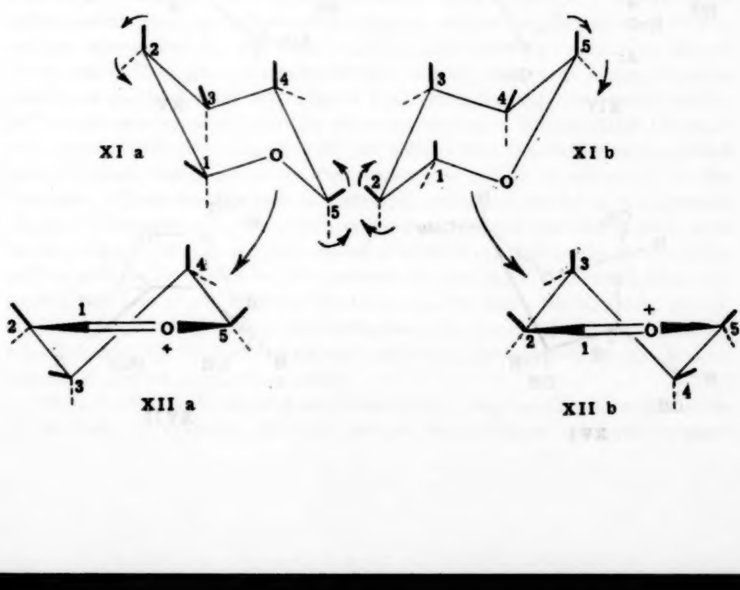
this to be attributable to "the tendency of a ring hydroxyl-group orientated perpendicular to the plane of the ring (B1) to go to an equatorial position (3B) upon dissociation." If the distribution of the two conformations in the amylose chains were determined, these results could well lead to a clearer understanding of the different modes of action of the enzymes concerned in starch synthesis and degradation; one wonders, too, whether some other polysaccharides may not also contain sugar units with more than one ring conformation.

Foster, Martlew & Stacey (109) have shown that methyl β-D-glucosaminide hydrochloride is deaminated by aqueous sodium nitrite far more rapidly than is the α-anomer and have suggested that this is attributable to the α-methoxyl group (which is the only bulky axial group in these compounds) hindering the approach of the reagent. The conformations of D-glucosamine and its derivatives have been shown by Fodor & Otvös (110) to explain the ease with which an acetyl group will migrate from position 3 to position 2 and vice-versa.

Foster & Overend (111) and Edward (112) have offered interesting explanations of the marked differences in the rates of acidic hydrolysis of various pyranosides. The former authors gave reasons for comparing the hydrolytic process with a conformational oscillation (VII→IX); they noted that such an oscillation would involve a considerable increase in the nonbonded interactions and consequently meet with resistance proportional to the number of equatorial substituents in the preferred conformation (VII) in

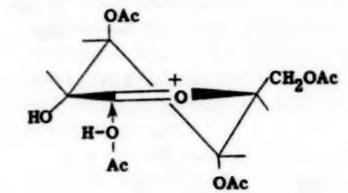
the glycoside. Such resistance should be greater for methyl glucosides than for other methyl pyranosides and these have, in fact, the lowest rate of hydrolysis. The observed reduction in the hydrolysis constant when methyl ether groups are introduced is attributable to the increased bulk of the substituents, resulting in greater nonbonded interactions; conversely, deoxy-groups increase the hydrolysis constant (111). Edward (112) depicted the course of the hydrolysis as X→XIII; he believed that the detachment of the methoxyl group from the chair form (XIa or XIb) would give the "half-chair" form (XIIa or XIIb), analogous to the conformation suggested earlier (113) for cyclohexene. This change would necessitate small rotations about the C-2—C-3 and C-4—C-5 bonds and would, therefore, be influenced by the configuration, and the size of the substituent, at each of the positions 2, 3, 4, and 5 (112). The hypothesis, like that of Foster & Overend (111), was observed (112) to be in accord with the known orders of decreasing stability in the sequences: (*a*) heptoside > hexoside > pentoside, in cases where the only structural differences are in the substituents at C-5 [$CH(OH) \cdot CH_2OH$; CH_2OH; H], and (*b*) glycoside > 2(or 3)—deoxy-glycoside > 2,3-dideoxy-glycoside. Solvent interaction was invoked (112) in order



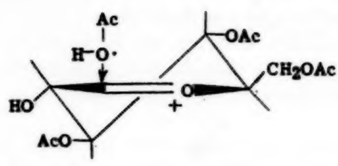


to explain why 6-deoxy-glycosides are more stable than their parent glycosides. From a consideration of the changes X→XIII, Edward (112) predicted also that there should be decreasing stability in the following sequence of methyl glycosides, when all are in the C1 conformation: glucoside (no axial OH) >alloside, mannoside, galactoside (one axial OH) >altroside, guloside (2 axial OH groups, each opposing a hydrogen) >taloside (2 axial OH groups, opposing each other) >idoside (3 axial OH groups); the sequence determined experimentally to date is: glucoside >mannoside >galactoside >guloside. A predicted sequence for pentosides also agreed with the experimental evidence.

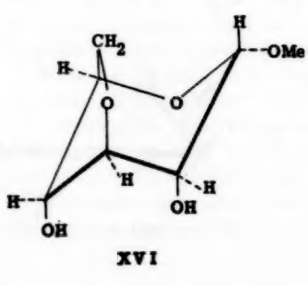
"Half-chair" conformations have been assigned also to intermediates in other reactions of pyranose derivatives. For example, 3,4,6-tri-O-acetyl-β-D-glucopyranosyl chloride was found (114) to undergo solvolysis in acetic acid to form 1,3,4,6-tetra-O-acetyl-α-D-glucopyranose as the main product. The much less reactive anomeric α-chloride also appeared to undergo solvolysis with extensive inversion of the anomeric center. These results were explained (114) on the basis of "half-chair" conformations of the cations; thus, the cation (XIV) from the β-chloride accepts an anion on the α-side of the ring, whereas the cation (XV) from the α-chloride is more open to attack on the β-side of the ring. The rates of anomerization and of dissociation at C-1 in α- and β-D-glucopyranose penta-acetates have likewise been explained (115) in terms of "half-chair" conformations of the cations. Closely related studies of substitutions at C-1 of pyranoses and their derivatives have been reported by Huber (116).



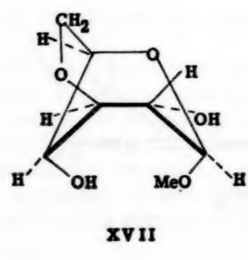
XIV



XV



XVI



XVII

Arguments based on conformational analysis have been advanced (117) to explain the action of methanolic hydrogen chloride on methyl 3,6-anhydropyranosides. Two conformations (XVI and XVII) are possible for methyl 3,6-anhydro-α-D-glucopyranoside; the requirement that the 5-membered ring should be nearly planar prevents any appreciable oscillation involving C-3, C-4, and C-5, so that it is the nonbonded interactions between the substituents at C-1 and C-2 and those on the planar ring which must mainly determine the favored conformation. Estimates were made of the extent of these interactions, from which it was concluded (117) that the most stable states of methyl 3,6-anhydro-2,4-di-O-methyl-D-glucopyranoside and -D-galactopyranoside are the β-boat forms, whereas in the case of methyl 3,6-anhydro-2,4-di-O-methyl-D-mannopyranoside the α-chair form is preferred. As experimental support for the hypothesis, it was shown (117) that methanolic and ethereal hydrogen chloride catalyzed an $\alpha \rightarrow \beta$ transformation in the glucoside and galactoside cases, but a $\beta \rightarrow \alpha$ transformation in the mannoside case. Other examples of the successful application of these principles were quoted. It was appreciated that some over-simplification of the situation was produced by neglecting dipole moments and hydrogen-bonding.

Several authors (118 to 123) have considered the theoretical aspects of reactions involving the opening of rings of the ethylene oxide type in anhydro-sugars. In the most recent of these papers, Overend & Vaughan (123) tabulated the experimental facts as far as nucleophilic reagents are concerned and summarized the earlier, rather divergent, opinions. They noted that Bose *et al.* (118) had drawn conclusions which were invalid when a greater range of reactions was considered and also that other factors besides electrostatic interactions must play a part in determining the nature of the products. Moreover, they doubted whether Cookson's theory (119), which was based on analogy with the cyclohexene oxide structure, could apply to highly-substituted anhydro-sugars because of the requirement that the carbon atoms carrying the oxygen bridge and their two neighbors should be coplanar. They gave examples to demonstrate that rings of the ethylene oxide type can be formed when one of the hydroxyl groups concerned is axial and the other equatorial, contrary to the conclusion of Reeves (100). Overend & Vaughan (123), like Angyal (120), considered that the conformation of the anhydro-sugar itself primarily determines the point of attack; it is the equatorial C—O bond which is ruptured preferentially by a nucleophilic reagent. Subsequently, the reaction product adopts the conformation in which nonbonded interactions are at a minimum; this conformation may well be different from that of the parent anhydro-sugar. However, when the conformation of the anhydro-sugar is not partly fixed, the dynamic stereochemical equilibrium makes the carbon-oxygen bonds of the anhydro-ring equally accessible to the reagent and nonbonded interactions in the product assume a greater importance (123).

Mills (124) has shown that conformational analysis offers an explanation of the order of stability of cyclic acetals derived from polyhydroxy-com-

pounds; this explanation is an alternative to the earlier theory (125) based on a planar zig-zag structure for the polyhydroxy-compound. It enables certain predictions of structure to be made which were not possible before; his prediction that arabitol should give the 1,3-2,4-, rather than the 2,4-3,5-, di-O-methylene acetal has since been verified experimentally (126). Conformational considerations have led Buchanan (121) to the conclusion that the optical center in the benzylidene group undergoes racemization when certain derivatives of methyl 4,6-O-benzylidene-α-D-galactoside are treated with alkali.

Although conformational analysis is a recent addition to the armory of the carbohydrate chemist, it has already made very useful contributions to the better understanding of a number of chemical reactions of carbohydrates. There can be little doubt that it will prove equally effective in studies of enzymic transformations in this field. For example, the anomerizations of methyl pyranosides which carry 3,6-anhydro bridges (p. 93) proceed with a rapidity and specificity reminiscent of enzyme reactions, and it is not inconceivable that transglycosylases may function by bridging the sugar molecules in a similar fashion, through their prosthetic groups. Quite recently, Bentley (127) has postulated that the oxidation of glucose by notatin proceeds by a mechanism which involves a rate-determining attack on the hydroxyl-group on C-1; this step is sterically hindered when the group is axial, but not when it is equatorial. It will be recalled that Magasanik, Franzl & Chargaff (128) found that *Acetobacter suboxydans* oxidizes a cyclitol only at an axial hydroxyl-group which has an appropriate equatorial hydroxyl-group in the *meta* position; it is now suggested (127) that the rate-determining step in this case is attack on the equatorial C—H bond and not on the axial hydroxyl.

Paper Electrophoresis of Carbohydrates

Paper electrophoresis is now almost as widely used as paper chromatography for small-scale separations of carbohydrates; to a large extent the methods are complementary. Reviews of the earlier work on paper electrophoresis have been prepared by Lederer (129), Parker (130), and Block, Durrum & Zweig (131); the last-named authors have considered quantitative aspects. Domenech *et al.* (132) have made a statistical determination of the limits of sensitivity. The method is clearly admirable for separating carbohydrates according to their acidic, neutral, or basic character, but attention is here confined to separations between neutral carbohydrates.

It is usual in paper electrophoresis of neutral carbohydrates to confer charges on the molecules by the formation of borate complexes at pH 8 to 10. Under these conditions, separations which are difficult or impossible by paper chromatography can often be achieved with ease, provided that the components of the mixtures possess appropriate structural differences (133 to 137). For example, Foster (133) compared electrophoresis in borate at pH 10 (900 volts) with the benzylamine paper chromatographic technique

(138); the former was much the better method for maltose-isomaltose, maltose-cellobiose, and gentiobiose-isomaltose mixtures, while the latter was the more suitable for laminaribiose-isomaltose mixtures and for the homologous series of 1,4-linked glucosaccharides. Using a high voltage electrophoresis technique in order to minimize diffusion effects (139, 140), Gross (137) has developed a method for the long-sought microseparation of acyclic polyhydroxy compounds; at 2000 volts in 0.05*M*-borate buffer (pH 9.2) erythritol, sorbitol, mannitol, and dulcitol were completely separated in 3 hr. Under similar conditions, erythritol, sorbitol, and mannitol were separated from glucose, fructose, mannose, and sorbose.

Recent extensions of the borate electrophoresis technique into the polysaccharide field represent an important advance. In 1954, Northcote (141) was able to fractionate mixtures of polysaccharides in borate buffer (pH 9.2) using a Tiselius apparatus; the borate was essential for satisfactory results, even with polysaccharides containing only *trans*-vicinal hydroxyl groups. Yeast glycogen and several starches gave at least two bands, but yeast mannan, ivory nut mannan, and rabbit liver glycogen were apparently homogeneous. Subsequently Preece & Hobkirk (142) separated a mixture of cereal polysaccharides by electrophoresis on paper. More recently, Foster, Newton-Hearn & Stacey (143) have reported the behavior of a wide range of amylosaccharides during paper electrophoresis in alkaline borate. They encountered difficulties with amylose, but not amylopectin, as a result of adsorption on the paper; with trace amounts of amylose, there was complete adsorption at the starting line and, with larger amounts, there was appreciable streaking. The addition of urea suppressed the adsorption and better separations were then possible. It is interesting that amylose and amylopectin have constant mobilities on paper, whether they are run together or separately (143), whereas in free solution they do not (141). Together with Mould & Synge's (144) elegant demonstration that amylosaccharide-iodine complexes, produced by hydrolysis of amylose, or by controlled synthesis with phosphorylase (145), can be fractionated according to molecular size by electrophoresis in agar jelly, these developments would seem to offer a promising new approach to the problems of polysaccharide purification and identification.

Detailed studies (133, 146) of the electrophoretic mobilities of glucose derivatives have shown that, in borate buffer at pH 10, complexes are formed which involve the hydroxyl groups at the following positions: (*a*) 3,6 (or 3,5) and 5,6 in glucofuranosides; (*b*) 4,6 in glucopyranosides; (*c*) 1,2 in the α-pyranose and α-furanose forms and 2,4 and 4,6 in the *aldehydo*-forms in reducing glucose derivatives (which do not have a fixed ring structure). It is interesting that the principal complexes in class (*c*) are those involving the *aldehydo*-forms (133), particularly since the pairs of hydroxyl groups (2,4 and 4,6) engaged by the borate correspond to the types (βC and β) which are known to favor cyclic acetal formation in acyclic polyhydroxy compounds (125). It is apparent that in an alkaline borate solution of a glucose deriva-

tive all the hydroxyl groups are probably involved in complex formation to a greater or smaller extent, whereas in aqueous boric acid only the 1,2-hydroxyl groups in the α-pyranose and α-furanose forms are involved (146, 147). Nevertheless, the marked differences in the affinities of the various hydroxyl pairs for borate make paper electrophoresis a most valuable tool in structural studies. It is likely that yet another type of interaction occurs between amylose and alkaline borate, and this may arise from entrapping of borate ions in the helical chains of the polysaccharide (143). The wide difference between the mobilities of methyl α- and β-D-glucopyranoside has been attributed to steric hindrance to the approach of the borate ion by the axial methoxyl group in the α-anomer (146). Evidence has been obtained by Bell & Northcote (136) that fructose and its derivatives may become "fixed" in the pyranose form by reaction with alkaline borate at the 4,5-position. The information gained from these electrophoretic studies is likely to be of considerable value in deciding whether a given mixture of carbohydrates might be separated on a macro-scale using borate buffers in conjunction with columns of anion-exchange resins (148 to 151) or of charcoal (152, cf. also 153).

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CHEMISTRY OF THE LIPIDES

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Since the chemistry of the lipides was reviewed by Hilditch (1) in 1953, new and interesting fatty acids have been discovered and the mechanisms of autoxidation and of fat synthesis have become better understood, as has the relationship between fatty acid composition and species. In this review it is proposed to emphasise items selected from publications that have become available during the year ending November, 1955.

Fatty Acids

Estimation and separation.—As a means of separating fatty acid mixtures paper chromatography has continued to prove a useful field of research. To separate fatty acids within the range C_2 to C_{24}, filter paper impregnated with high boiling point petroleum hydrocarbon as the stationary phase has been used with the following solvents for the mobile phase: aqueous methanol (2, 3), methanol-acetone mixtures (2), and aqueous acetic acid (4, 5). According to Kaufmann & Nitsch (4) acetic acid-water mixtures give sharper separations than the diluted alcohols and may even be used to separate oleic and linoleic acids (6). To secure reproducible results Spiteri (5) has emphasised the need for care in the method of impregnating filter paper with the stationary phase. He also showed the sensitivity of the method by providing evidence for the occurrence of behenic and lignoceric acids in olive oil. To locate the acid, bromocresol green (2), ammoniacal silver nitrate (5), copper acetate-potassium ferrocyanide (4), and rhodamine B (4) have been used. However, the most sensitive technique according to Ashley & Westphal (3) is to treat the developed paper with lead acetate and subsequently to convert the lead salts to sulfide.

Other effective systems for the separation of higher fatty acids include the use of filter paper impregnated with a stationary phase consisting of potash alum solution and carbon tetrachloride:methanol:concentrated ammonia solution in the v/v ratio 81:12:1 (7) for the mobile phases. Another system involves silicone-impregnated paper with a mobile phase consisting of an acetone, water, and cyclohexane mixture (8).

The hydroxamates of the fatty acids from C_6 to C_{18} have been separated by Micheel & Schweppe (9) on cellulose acetate paper with a mixture of ethyl acetate, tetrahydrofuran, and water. One of the difficulties with chromatographic techniques for fatty acids has been the nonresolvability of saturated and unsaturated acids, such as palmitic and oleic acids. This difficulty has now been overcome by Inouye, Noda, & Hirayama (10) by using the mercuric acetate addition compounds of unsaturated fatty acids.

Either 1,2,3,4-tetrahydronaphthalene (Tetralin) or petroleum hydrocarbon is used as the stationary phase, the corresponding mobile phase solvents being aqueous methanol and methanol acetic acid respectively. The position of the fatty acids is given by diphenylcarbazone. The absorption spectra of the carbazone complexes offer the possibility of identification and of quantitative measurement of the amounts of unsaturated fatty acids. The sensitivity of the method was shown by the detection of tetradecenoic acid in olive oil and of hexadecenoic acid in linseed oil, neither of these acids having previously been found in these sources.

In the sphere of column absorption chromatography, the reversed-phase, paraffin coated kieselguhr-column of Howard & Martin (11) with aqueous acetone as the mobile phase has found useful application. Silk & Hahn (12) used this technique to determine the composition of the unsaturated acids of pilchard oil as well as for the isolation of hexadec-6,9,12,15-tetraenoic acid (13). Crombie, Comber & Boatman (14) found that the microdetermination of the fatty acid composition, using Howard-Martin columns, gave results in good agreement with those obtained by other techniques. Certain pairs of saturated and unsaturated acids, such as palmitic and oleic, are inseparable by this technique. To overcome this difficulty the analysis is carried out first on the total mixed fatty acids and then on the saturated acids separated by the Bertram method, and the unsaturated acids determined by difference.

Green, Howitt & Preston (15) found that polythene powder columns provide a simple and effective reversed-phase chromatogram without the disadvantage of loss of efficiency through removal of the liquid paraffin phase as may occur with the Howard-Martin system.

Nijkamp (16) quantitatively separated the fatty acids from C_2 to C_{24}, using specially prepared silica gel columns saturated with methanol and containing bromothymol blue neutralized with ammonia. For the mobile phase isooctane saturated with 95 per cent methanol was used, the acids appearing as sharp bands. Ikeda, Webb & Kepner (17) separated the orange coloured *p*-phenylazophenacyl esters of the C_2 to C_{18} fatty acids on activated silica columns, using 50 per cent benzene in Skellysolve B as the developing solvent. At the higher molecular weight levels there was only a poor separation of fatty acids which differed by two carbon atoms, but a good separation resulted when the fatty acids differed by four carbons.

To characterise the component fatty acids obtained by ester fractionation analyses, Davenport (18) used the hydroxamic acid derivatives which he separated by chromatography on cellulose columns. From a mixture of C_{18} unsaturated acids it was shown that the pure hydroxamic acids of oleic, linoleic, and linolenic acids could be recovered.

Two groups of workers have independently examined chromatography as a method of separating bromo fatty acids. Moretti & Polonovski (19) used celite or cellulose columns to separate the brominated fatty acids. Dibromostearic acid, followed by stearic acid, was eluted with light petroleum, tetrabromostearic acid with ethyl ether, and, finally, hexabromostearic acid

with dichloroethylene at 50°C. Howton (20) has shown that bromo esters are easily and cleanly separated by graded elution on alumina columns whereas the esters of unsaturated acids are separated with difficulty. This observation is likely to be of value in the investigation of complex mixtures of the unsaturated fatty acids present in natural fats.

The gas-liquid chromatography technique used by James & Martin (21) for the separation of fatty acids, including isomers up to C_{11}, has been extended by Cropper & Heywood (22) to the estimation of the methyl esters of C_{12} to C_{22} fatty acids. In the stationary phase the silicone oil is replaced by high vacuum silicone grease, and the separation is made in a stream of nitrogen at 230°C. under reduced pressure. The amounts of esters in the effluent nitrogen are measured by the thermal conductivity method suggested by Ray (23). Dijkstra *et al.* (24) have since shown that the operation may be carried out more satisfactorily at atmospheric pressure. James & Martin (25) suggested a new method, based on the measurement of density change, which permits the detection of 0.06 μg. of substance per ml. of nitrogen and thereby provides the most sensitive means so far devised for the quantitative microdetermination of volatile constituents. Eventually because of speed, accuracy, and sensitivity, it is most probable that gas-liquid chromatography will replace the existing procedures for analysis of fatty acid mixtures.

Silk & Hahn (12) have shown that concentrates of highly unsaturated acids of pilchard oil prepared by urea fractionation are richer in C_{16} and C_{18} acids but contain less of C_{20} and C_{22} unsaturated acids than concentrates prepared by the lithium soap-acetone method. McElroy *et al.* (26) used the urea complexes to fractionate the fatty acids of bovine testicular lipides. In comparing the precipitates formed successively at 5°, −20°, and −75°C. in ethanol, it was found that docosahexaenoic acid concentrated in the −20°C. precipitate and eicosahexaenoic acid in the filtrate at −75°C. The latter acid has not hitherto been recorded as a constituent of natural fats. To separate fatty acid mixtures Rigamonti & Riccio (27) used the differences in equilibrium constants of the reaction between urea and fatty acids partitioned between light petroleum and water. Narayan & Kulkarni (28) have shown that the formation of urea fatty acid complexes in ethanol-water media is highly sensitive to the concentration of urea and of water, while Sakurai (29) found that the segregation of stearic and oleic acids by urea complex methods is more effective when applied to the methyl esters than to the free acids.

Bergström & Pääbo (30) used performic acid hydroxylation followed by chromatography to separate saturated and mono-unsaturated fatty acids. Hofmann *et al.* (31) similarly separated the unsaturated acids from branched-chain and saturated acid in small samples of bacterial lipides. These samples were also assayed for lactobacillic acid, using microbiological methods.

The spectrophotometric methods of analysis of fats were critically reviewed by Herb (32), while Kolb & Brown (33) have provided further data on the solubility of fatty acids as a guide to their separation by low tem-

perature crystallization from solvents. Hammond & Lundberg (34) have developed equations for the calculation of molar refraction, molar volume or density, and refractive index for liquid fatty acids and esters in relation to chain length and unsaturation. These equations are useful for identification and in the determination of structure or of purity.

Synthesis.—Linstead and collaborators have used anodic cross-coupling extensively for the synthesis of unsaturated acids. This procedure apparently does not involve stereomutation (35). *Cis*- and *trans*-tetracos-15-enoic acids have been synthesised from oleic and elaidic acids, respectively, by coupling with excess methyl hydrogen suberate. The *cis* acid has been found to be identical with nervonic acid from cerebrosides and with selacholeic acid from shark liver oil (36). The electrolysis of palmitoleic acid with methyl hydrogen succinate has provided a stereospecific synthesis of *cis*-octadec-11-enoic acid (37) identical with the *cis*-vaccenic acid, the haemolytic factor of horse brain, and the principal unsaturated acid of *Lactobacillus arabinosis* and *Lactobacillus casei.*

A stereospecific synthesis of vaccenic (*trans*-octadec-11-enoic) acid was accomplished by the bromination and debromination of threo-11,12-dihydroxystearic acid (37). The latter was prepared by electrolysis of methyl hydrogen succinate and threo-9,10-dihydroxypalmitic acid obtained by the performic acid oxidation of palmitoleic acid.

Electrolysis of threo-9,10-dihydroxystearic acid with excess benzyl hydrogen succinate yielded threo-11,12-dihydroxyarachidic acid (38) which was identical with the dihydroxy acid obtained by the performic acid oxidation of the natural eicos-11-enoic acid present, particularly in the liquid seed wax of *Simmondsia california.* The naturally occurring acid, therefore, has a *cis* configuration.

It has been shown that anodic coupling is also applicable to acetylenic acids, provided the triple bond is well removed from the carboxyl group. Thus octadec-6-ynoic acid, identical with the naturally occurring tariric acid, has been prepared from methyl hydrogen dodec-6-yne dioate and octanoic acid. Partial hydrogenation of octadec-6-ynoic acid with Lindlar lead-palladium catalyst yielded *cis*-octadec-6-enoic acid identical with natural petroselenic acid (39). Stearolic acid (octadec-9-ynoic) has been prepared by anodic synthesis by coupling acetylenic acids of the type

$$HO_2C \cdot (CH_2)_n \cdot C \vdots C \cdot (CH_2)_n \cdot CO_2H$$

(where $n = 3$ or 4) first at one end of the molecule with a monocarboxylic acid and then at the other with a half ester. Partial reduction using Lindlar catalyst of the stearolic acid thus formed gave oleic (octadec-*cis*-9-enoic) acid in high yield (40).

Further examples include the anodic synthesis by Mislow & Steinberg (41) of chaulmoogric acid (identical with the naturally occurring acid), using (+)-2-cyclopentene-1-acetic acid. Also anodic coupling has been used by Milburn & Truter (42) for the synthesis of the *iso*- and *anteiso* acids of wool

wax. For this purpose, (+)-4-methyl hexanoic acid [derived from naturally occurring (−)-2-methyl butanol] and 4-methyl butanoic acid respectively were coupled with the half esters of the appropriate dibasic acids (42).

By the anodic synthesis of L-α-hydroxy acids from

$$C_2H_5\cdot O\cdot OC\cdot CH(OAc)\cdot CH_2\cdot COOH$$

[derived from naturally occurring (−)-malic acid] and the appropriate straight chain acids, Horn & Pretorius (43) showed that the naturally occurring α-hydroxy acids of wool grease and their derived diols, as well as phrenosinic acid from brain, have the D configuration.

The examples given show the flexibility of the anodic synthesis techniques. However, the extension of the method to the preparation of the naturally occurring polyethenoic acids has not yet been achieved.

The synthesis of (±)-ricinoleic acid (12-hydroxyoctadec-9-enoic) acid has been accomplished independently by Crombie & Jacklin (44) and by Kendall, Lumb & Smith (45). The latter used as the key intermediate 1-chloro-10,10-dimethoxydec-7-ene prepared by the condensation of 1-chloro-7-octadecyne and 1-bromo-2,2 dimethoxyethane, followed by Lindlar reduction. Extension of the acetal chain by the addition of *n*-hexyl magnesium bromide gave the unsaturated secondary alcohol which, by malonic acid synthesis followed by hydrolysis, decarboxylation, and esterification with methyl alcohol, gave (±)-methyl ricinoleate with infrared absorption and other characteristics corresponding with those of the natural D acid. Crombie & Jacklin (44) used the Reformatski reaction between propargyl bromide and heptaldehyde to give 4-hydroxydec-1-yne which, by chain extension, was converted to 1-chloro-10-hexadec-7-yne. From the latter, by malonic ester synthesis, (±)-ricinostearolic acid identical with that obtained by bromination and debromination of natural D-ricinoleic acid was prepared. Partial hydrogenation gave (±)-ricinoleic acid.

Ximenynic acid (octadec-11-en-9-ynoic acid), which occurs in the kernel oils of three *Ximenia* species, has been synthesised by Grigor, MacInnes & McLean (46) from ricinostearolic acid derived from castor oil. The methyl ester was converted to methyl 12-chloro-stearolate with thionyl chloride which, on hydrolysis with methanolic potassium hydroxide, gave octadec-11-en-9-ynoic acid with a *trans* olefinic link, as shown by infrared absorption.

Badger, Rodda & Sasse (47) have shown that the desulphurisation of γ-2-thienyl-, γ-5-ethyl-2-thienyl-, γ-5-heptyl-2-thienyl-, and γ-5-undecyl-2-thienyl-butyric acids gives respectively *n*-octanoic, *n*-decanoic, *n*-pentadecanoic, and *n*-nonanoic acids in good yields. The method can also be extended to the synthesis of branched chain fatty acids.

Isolation and constitution.—Holman & Hanks (48) found that the lower boiling point fraction of the methyl esters of *Sebestiana linguistra* seed oil, after purification by displacement chromatography, yielded a C_{12} acid which on hydrogenation was converted to lauric acid. From the mean unsaturation and ultraviolet absorption data it was concluded that the acid

was dodec-2,4-dienoic. This acid had not hitherto been isolated from natural sources, though the lower homologue, deca-2,4-dienoic acid, was isolated by Hilditch (49) from the seed oil of *Sapium sebiferum.*

The constitution of α-kamlolenic acid and the main constituent of Kamala (*Mallotus philippinensis*) seed oil has now been finally settled as 18-hydroxyoctadeca-9,11,13-trienoic acid, on the following grounds: (*a*) on reduction three molecules of hydrogen are absorbed giving 18-hydroxyoctadecanoic acid (50), (*b*) the ultraviolet absorption spectrum indicates the presence of a conjugated triene system (51), and (*c*) methyl 8-formyl octanoate has been isolated after the ozonolysis of methyl kamlolenate (52, 53). Ultraviolet and infrared absorption studies by two independent groups of workers (54, 55) revealed conjugated *trans-trans-cis* or *cis-trans-trans* bonds similar to those of α-elaeostearic acid while the β-kamlolenic acid obtained by iodine-catalysed ultraviolet irradiation of the α-form showed all *trans* bonds similar to those of β-elaeostearic acid. α-Kamlolenic acid occurs wholly or partly as a polyester (56).

Ximenynic acid, originally found in *Ximenia* species, has been discovered by Hatt & Szumer (57) in the seed fat of two Australian members of the Santalaceae. These workers predicted that santalbic acid, isolated from the seed fat of the related *Santalum album* and supposed by Madhuranath & Manjunath (58) to be a nonconjugated octadecatrienoic acid, would prove to be identical with ximenynic acid. This has now been established by Gunstone & McGee (59).

Lighthelm (60) isolated from *Ximenia caffra* Sond. an unusual unsaturated fatty acid which, on hydrogenation, gave a monohydroxystearic acid. The infrared spectrum corresponded to that of ximenynic acid, while the evidence from lithium aluminium hydride reduction and from alkali isomerisation was consistent with the close proximity of the hydroxyl group to the enyne system. This evidence, together with degradative oxidation, indicated the structure $CH_3\cdot(CH_2)_5\cdot CH{:}CH\cdot C\vdots C\cdot CHOH\cdot(CH_2)_6\cdot COOH$. Gunstone (61) has examined the constitution of vernolic acid, the main constituent of *Vernonia anthelmintica* seed oil. The structure was established from the quantitative determination of the epoxy group and from the dihydroxy acid formed by treatment of the oil with acetic acid followed by alkaline hydrolysis. Degradative oxidation showed the dihydroxy acid to be 12,13-dihydroxyoctadec-9-enoic. Hence the original epoxy acid has the structure 12,13-epoxyoctadec-9-enoic acid.

Verma *et al.* (62) recently claimed that the action of diazomethane on stearolic acid gave an acid of the structure

$$CH_3(CH_2)_7\cdot \underset{\diagdown\ \underset{CH_2}{} \ \diagup}{C{=\!=}C}\cdot(CH_2)_7COOH,$$

proposed by Nunn (63) for sterculic acid, a constituent of the kernel oil of *Sterculia foetida.* The acid, however, differed considerably from sterculic acid

as shown by its infrared spectrum. To account for this difference in properties as well as for the chemical behaviour of sterculic acid Verma *et al.* (62) proposed the structure

$$CH_3(CH_2)_5\cdot \underset{\diagdown\;CH_2\;\diagup}{CH—CH}\cdot CH{:}CH\cdot(CH_2)_7COOH.$$

Further comment on this work must await publication of the experimental details. Meanwhile Dijkstra & Duin (64) consider that the following evidence is in agreement with Nunn's formula: (*a*) the infrared absorption data before and after hydrogenation of sterculic acid parallel the displacement which follows the increased ring strain of cyclopentene as compared with that of cyclopentane; (*b*) the Halphen reaction and the band at 1009 cm.$^{-1}$ are given by the *S. foetida* oil as well as by kapok seed oil (which contains a C_{18} analogue of sterculic acid) but not by cyclopropane derivatives; and (*c*) the structure proposed by Nunn (63) is more compatible than that of Verma *et al.* (72) with the rapid polymerization of *S. foetida* oil.

Hofmann & Tausig (65) have shown that phytomonic acid, isolated from the lipides of *Agrobacterium* (*Phytomonas*) *tumefaciens*, is not 10- or 11-methylnonanoic acid as supposed by Velick (66) but identical with lactobacillic acid, previously isolated from *Lactobacillus arabinosis* (67) and *L. casei* (68). A comparison of the infrared absorption spectra of lactobacillic acid and of dihydrosterculic acid with those of synthetic *trans*-9,10- and 11,12-methyleneoctadecanoic acids offered convincing evidence for the cyclopropane structure of these acids. Lactobacillic acid resembled most closely, in its long spacing intensity distribution, *trans*-11,12-methyleneoctadecanoic acid (69).

Hopkins & Chisholm (70), in continuation of work on sources of Δ^{11}-eicosenoic acid, have now shown that this acid occurs to the extent of 4 to 11 per cent in the following: seed oils of rutabaga (*Brassica napobrassica* Mill), and of french weed (*Thlaspi arvense* L), dogfish (*Squalus acanthias* L) liver oil, and the blubber oil of the whale (*Delphinapterus leucas Pallis*).

Klenk & Dreike (71), after using the techniques of alkali isomerisation and ozonolysis previously described (72), considered that the results obtained for the C_{18}, C_{20}, and C_{22} unsaturated acids of ox liver glycerophosphatides were consistent with the presence of the following constituents: linoleic, linolenic, $\Delta^{5,8,11}$- and $\Delta^{8,11,14}$-eicosatrienoic, $\Delta^{5,8,11,14}$- and $\Delta^{8,11,14,17}$-eicosatetraenoic, $\Delta^{5,8,11,14,17}$-eicosapentaenoic, $\Delta^{7,10,13,16,19}$-docosapentaenoic, and $\Delta^{4,7,10,13,16,19}$-docosahexaenoic acids. The methods used, however, do not exclude the possibility that one or other of the C_{20} triene or tetraene acids may be absent. It is also possible that C_{20} diene and C_{22} acids less unsaturated than pentaene may be present.

Klenk & Lindlar (73, 74) improved the earlier techniques by introducing countercurrent extraction to resolve the fractions into essentially pure acids or mixtures of closely related acids. By this means they have determined the

constitution of the glycerophosphatide fatty acids of human brain with considerable certainty as follows: $\Delta^{5,8,11,14}$-eicosatetraenoic, $\Delta^{5,8,11}$- and $\Delta^{8,11,14}$-eicosatrienoic and $\Delta^{11,14}$-eicosadienoic, $\Delta^{4,7,10,13,16,19}$-docosahexaenoic and $\Delta^{7,10,13,16}$-docosatetraenoic. In addition, the presence of $\Delta^{4,7,10,13,16}$-docosapentaenoic acid and probably of docosatrienoic was indicated. These results somewhat simplify the picture earlier obtained by Klenk & Bongard (75) in which the $\Delta^{4,7,10,13}$-docosatetraenoic and $\Delta^{7,10,13,16,19}$-docosapentaenoic acids were also included. The general conclusions to be reached are that the polyene acids so far described follow the divinyl methane pattern and that they can be built up from linoleic and linolenic acids by chain extension.

It has been generally assumed that eicosatetraenoic (arachidonic) acid in animal fats has the $\Delta^{5,8,11,14}$ structure (76). This would be consistent with its biological formation by the addition of acetate to linoleic acid which has been demonstrated by Mead, Steinberg & Howton (77). Shimo-oka & Toyama, however, from the results of degradative oxidation with permanganate in acetone solution, claim that the eicosatetraenoic acid in ox liver (78) and in pig liver lipides (79) has a $\Delta^{4,8,12,16}$ structure. The ultraviolet absorption spectrum, following alkali-isomerisation of the acid described by Shimo-oka & Toyama (79), corresponds to that of arachidonic acid and is difficult to interpret on the basis of a $\Delta^{4,8,12,16}$ structure. It is important that the structure of C_{20} tetraene acids should be further investigated to determine whether there are, in fact, two kinds present in animal fats.

Baruch's (80) proof of the structure of oleic acid as octadec-9-enoic, involving the conversion of sterolic acid to 10-oxostearic acid, has been invalidated by Gunstone (81), who pointed out that 10-oxostearic acid could have been derived by the hydration of octadec-9- or 10-ynoic acid.

Branched-chain fatty acids.—Although isovaleric acid has long been known to occur in the fats of dolphins and the porpoises (82), it was not until 1951, following the discovery by Hansen & Shorland (83) of a branched-chain fatty acid in butterfat, that a series of higher molecular weight branched-chain fatty acids was found in some animal fats.

The branched-chain fatty acids of animal fats, so far as can be ascertained, are saturated and appear to consist mainly of the odd-numbered (+) *anteiso* acids of which the C_{13} (84), C_{15} (85, 86, 87), and C_{17} (88, 89, 90) members have been identified, and of even- and odd-numbered *iso*-acids of which the C_{13} (84), C_{14} (91), C_{15} (85, 86, 87), C_{16} (92), and C_{17} (88) members have been isolated. In addition, there have been isolated a C_{18} branched-chain acid [which is probably 10-methylheptadecanoic acid (93)], C_{17} (94), and C_{18} acids with two side methyl groups (95), and also a C_{20} (96) acid containing two or three side methyl groups.

The exact amounts of branched-chain acids in animal fats are not known accurately; nevertheless there is some evidence to suggest that collectively they form more than 1 per cent of the total fatty acids of some ruminant fats. Observing that the fractionating column used sharply separated the methyl esters of C_{15} acids, Shorland, Gerson & Hansen (87) estimated that the C_{15} acids formed 1.6 per cent of the total fatty acids of butterfat, and, that of

these, 0.43 per cent was (+)-12-methyltetradecanoic and 0.37 per cent was 13-methyltetradecanoic acid.

n-*Odd-numbered carbon fatty acids.*—Within the last two years it has been established that *n*-odd-numbered carbon fatty acids occur in natural fats. Hansen, Shorland & Cooke found that hydrogenated mutton fat contained 0.15 per cent of *n*-pentadecanoic acid (97) and 1.2 per cent of *n*-heptadecanoic acid (98), while Shorland, Gerson & Hansen found 0.82 per cent of *n*-pentadecanoic (87) and 0.05 per cent of *n*-tridecanoic acid (84) in unhydrogenated butterfat. Subsequently, Morice & Shorland (99) showed that 0.28 per cent of *n*-pentadecanoic and 0.17 per cent of *n*-heptadecanoic acids were present in shark liver oil.

Following the discovery of *n*-odd-numbered saturated fatty acids in animal fats, Shorland & Jessop (100) isolated from lamb caul fat Δ^9-heptadecenoic acid and, in addition, obtained evidence for the occurrence of Δ^9-C_{13} and -C_{15} acids. This result is perhaps not surprising as Appel *et al.* (101) had earlier demonstrated that *n*-odd-numbered saturated fatty acids, within the range C_9 to C_{19}, when fed, were deposited partly as the corresponding Δ^9-unsaturated acids.

Trans-*unsaturated acids in animal fats.*—Hartman, Shorland & McDonald (102) have shown that whereas *trans* acids are invariably present in substantial amounts (3.5 to 11.2 per cent) in the depot fats of ruminants they are usually absent from those of nonruminants. They considered, therefore, that *trans* acids were produced exogenously through hydrogenation of the dietary unsaturated acids by rumen microorganisms and not endogenously by action of oxidases as suggested by Swern, Knight & Eddy (103). In support of this hypothesis they showed that whereas pasture fat, consisting mainly of linolenic acid, contained not more than traces of *trans* acids, the fatty acids in sheep rumen contents contained 9 per cent of these acids.

Garton & Oxford (104) suggested that the rumen bacteria might contain *trans* acids. This is possible, but consideration of the available evidence suggests that most of the acids in rumen fluid are extraneous with regard to the bacteria. This is confirmed by Shorland, Weenink & Johns (105) who effectively hydrogenated linolenic acid with filtered rumen fluid (low in fat and free of *trans* acids) with the formation of 15 per cent *trans* acids. Further evidence of the connection between *trans* acid formation and the action of microorganisms on unsaturated acids was obtained by finding 21.0 per cent of *trans* acids (102) in the depot fat of the marsupial *Setonix brachyurus* (quokka) which Moir *et al.* (106) have recently shown to possess a ruminant-like digestion. The relatively high content of *trans* acids in the quokka appears to be related to the fact that the dietary polyene acids have not been so completely hydrogenated as in ruminants, in which the acids are largely reduced to the saturated stage.

Ruminant Metabolism and Fat Formation

It is now established that the volatile fatty acids arising from the breakdown of carbohydrates and proteins by microorganisms in the rumen con-

sist mainly of acetic, with lesser amounts of propionic, together with butyric, valeric, and hexanoic acids (107, 108). In addition, there occur the branched-chain acids, isobutyric, isovaleric, and (+)-2-methylbutyric which are believed to be derived from valine, leucine, and isoleucine respectively (108). The isolation of a series of even- and odd-numbered saturated *iso* acids and odd-numbered saturated *anteiso* acids together with *n*-odd-numbered acids saturated and unsaturated, earlier described, is in accord with the operation of the Lynen (109) fatty acid cycle involving the addition of acetate to isobutyric, isovaleric, (+)-2-methylbutyric, and propionic acid respectively. These acids are provided by the microbiological breakdown of the carbohydrates and proteins in the rumen. The proportion of the branched-chain and *n*-odd-numbered acids will naturally depend on the relative rates of synthesis and breakdown and on the alternative pathways for utilizing the precursors. Black & Kleiber (110) have indicated that propionate, apart from being glycogenic, follows several pathways, so that presumably only a fraction would be utilized for the synthesis of higher *n*-odd-numbered fatty acids. According to Zabin & Bloch (111) isovaleric acid is utilized in preference to acetate for cholesterol synthesis.

Both odd- and even-numbered *iso* acids are found in ruminant fats, but in wool wax, according to Weitkamp (112), even-numbered *iso* acids, only, are found. This might be explained by the utilization of isovaleric acid mainly for cholesterol formation since wool wax contains about 30 per cent of this constituent.

Ruminant digestion is not essential in the formation of branched-chain and *n*-odd-numbered fatty acids. Morice & Shorland have found in shark liver oil C_{15} and C_{17} *n*-odd-numbered fatty acids (99) as well as C_{16} and C_{17} *iso* and *anteiso* acids (113). Nor are branched-chain fatty acids necessarily derived from the branched-chain amino acids. Wüersch, Huang & Bloch (114), for example, have demonstrated how branched-chain fatty acids could be derived from the condensation of acetate molecules.

One of the most important features of ruminant metabolism and fat formation, already referred to in connection with *trans*-acid formation, is the hydrogenation of the dietary unsaturated acids (115). This is responsible for more than just the formation of *trans* acids. It forms an effective barrier to the passage into the depot fats, from ingested food, of highly unsaturated acids, such as linolenic acid, which is the main fatty constituent of the pasture. In its place the depot fats are provided with stearic acid (105), conjugated and nonconjugated diene acids, and monoene isomers.

The recent work of Allen & Kiess (116) suggests that the hydrogenation process would also induce isomerisation of the double bonds, leading to a wide variety of positional isomers in addition to *cis* and *trans* modifications.

Fatty Acid Composition of Some Animal Fats

Recent information on the fatty acid composition of animal fats is summarized by Piskur (117), but it is still desirable to discuss some of the implications, especially the relationship between dietary and depot fats. It has

generally been assumed, mainly on the basis of the content of C_{20} and C_{22} unsaturated acids that the fatty acid composition of the fats of amphibia fall between those of fish and terrestrial mammals. However, dietary considerations must be taken into account.

Gunstone & Russell (118) have shown that whereas the fats of a captive crocodile fed on piglets contained only 3.8 per cent of C_{20} and C_{22} unsaturated acids, in the fat of species living in their natural environment on a fish diet 11 to 12 per cent of these acids were present (119). Similarly captive tigers and pumas, fed cow and horse flesh, deposited considerable proportions (12 to 13 per cent) of C_{18} polyene acids derived from horse flesh, but the same species in their natural state, living on sheep and deer with fats of relatively high stearic acid content and low C_{18} diene content, reflected these features in their depot fats (120). Fats of the chimpanzee also reflected the vegetarian diet in containing 8 per cent linoleic acid (121). The fatty acid composition of ostrich fat (122) from birds fed on maize recalls that of horses similarly fed (123), particularly in respect to the high content (17.1 per cent) of C_{18} diene acids. Garton & Duncan (124) found, as in their earlier experiments on whale oil, that pigs fed with equal parts of lard and cod-liver oil, up to the level of 50 per cent of the weight of the diet, deposited these fats largely unchanged. Considered collectively, the results just described support the view expressed by Shorland (125) that the fatty acid composition of the depot fats of all animals except ruminants is greatly influenced by the composition of the dietary fat.

Although dietary fatty acids containing 10 carbon atoms or less are not stored to any appreciable extent in the depot fats, Weitzel *et al.* (126) have now shown that small quantities of octanoic, decanoic, and somewhat larger quantities of dodecanoic acids are normally stored in the skin of rats, and that when octanoic and decanoic acids are fed as glycerides they form up to 7 per cent of the total acids in the fat of the skin. Similarly, undecanoic and undecylenic acids when fed, collect in the skin. It may be mentioned, however, that, as earlier shown by Visscher (127), undecanoic acid when fed may be stored in the depot fat to the extent of 24 per cent of the total fatty acids. It is interesting to note that traces of continuous series of volatile acids from C_2 to C_{10} have recently been found in ox perinephric fat (128).

Garton (129) has shown that whereas the mammary gland fat of a lactating cow contains about half as much of the lower volatile acids as milk fat with compensating proportions of palmitic and stearic acids, the fat from a nonlactating cow resembles depot fat, apart from its somewhat higher content of palmitic acid.

Synthesis of Triglycerides

Barry & Craig (130) synthesised symmetrical 1,3-diglycerides of palmitic and stearic acids by two different methods. The first method was to interesterify the methyl esters of the fatty acids with 1,3-dipropionoxyacetone diethyl mercaptal and then to hydrogenate. The second method was to oxidize allyl tetrahydropyranyl glycerol, to acetylate, and then to interesterify

with methyl esters of the fatty acids. Subsequently Porck & Craig (131) prepared the symmetrical diglycerides in good yield and in pure form by acetylation of 2 benzyl-glycerol, followed by interesterification with methyl stearate or palmitate.

Hartman (132), using the greater reactivity of the primary hydroxyls as compared with that of the secondary hydroxyl, has developed a general method for the preparation of mono-, di-, and triglycerides of known constitutions. The difficulty of preparing mono- and diglycerides by direct esterification was in obtaining a homogeneous solution of glycerol, fatty acid chloride, and chloroform. This has now been overcome by the addition of dimethyl formamide. In this way the difficultly synthesised triglycerides, oleopalmitostearin, stearo-oleopalmitin, and palmitostearo-olein, have been prepared in pure form and in good yield by direct three stage esterification of glycerol.

Glyceride structure.—Glyceride structure was adequately discussed in this series of reviews by Deuel (133) in 1950, and again by Hilditch (1) in 1953. Recent developments show that no final understanding has been reached in regard to the distribution of fatty acids in the glyceride molecules. In the simplest form, not found in nature, each fatty acid will appear as simple triglyceride. This is the reverse of the widely accepted Hilditch concept of even or widest possible distribution. In stearic-rich animal fats this involves subsequent hydrogenation of preformed oleo glycerides to account for the occurrence of substantial amounts of fully saturated glycerides (GS_3) in the presence of less than 66.7 per cent saturated acids (134). This modification of the theory was justified on the basis that in these stearic-rich fats the unsaturated glycerides were still assembled on an even distribution pattern (135). Some investigators, however (136, 137), have suggested that random distribution operates in stearic-rich animal fats, and more recently Kartha (138, 139) has assumed that a random pattern operates in all natural fats. If the melting point of a fat were raised unduly, however, by the inclusion of high proportions of saturated glycerides of high molecular weight, the third saturated acid that would otherwise enter to form GS_3 would be replaced nonselectively by an unsaturated acid. Kartha (139) considered that there were two defects in the procedures used by Hilditch: (*a*) the inclusion of products, such as GS_2OH, formed by hydrolysis of azelaoglycerides, such as GS_3 in the determination of this fraction by oxidation with permanganate in acetone; and (*b*) the inclusion of disaturated monounsaturated glyceride (GS_2U) in the most soluble fractions calculated as containing only diunsaturated monosaturated (GSU_2) and triunsaturated glycerides (GU_3). To remedy the above defects, Kartha (139) determined the GS_3 by crystallisation from solvents, as in the later investigations by Hilditch. The remaining glyceride types were determined by quantitative conversion to azelaoglycerides with permanganate in acetone (the hydrolysis being suppressed by the addition of acetic acid) and the quantitative precipitation of the monoazelao glycerides as magnesium salts, coprecipitation of diazelaoglycerides being allowed for by determination of saturated acid content (139). Such oxidation

techniques, however, cannot provide a description of the types of fatty acids involved, as is possible with the Hilditch crystallisation procedure coupled with ester fractionation analyses. Kartha's (139) experimental results showed close agreement with his own theory of restricted random distribution.

Cama *et al.* (140), in reply to Kartha's criticism, gave detailed evidence to support the view that his work does not invalidate the general findings of the Hilditch School and, that in particular, such mixtures as oleodistearin and oleolinolein used by Kartha as evidence of ineffective separation by crystallisation can, in fact, be effectively separated by this procedure. The polemic has been continued by Hilditch (141, 142) and by Vander Wal (143).

Luddy, Fertsch & Riemenschneider (144), using both Kartha's techniques and the Hilditch procedures, found similar results for lard, chicken fat, and cottonseed oil but not for palm oil. The glyceride structure of chicken fat conformed to restricted random or random distribution, but the results for all the remaining fats did not show a random, a restricted random or, indeed, an even distribution pattern. Absence of random distribution in animal fats is also indicated by Quimby, Wille & Lutton (145) who found that whereas tallows are composed mainly of 1-palmityl glycerides, lard contains mainly 2-palmityl glycerides. Contrary to Kartha's suggestions they found more GS_3 but less GS_2U than called for by chance proportions.

Reiser & Dieckert (146) introduced an isotopic dilution procedure to determine the GS_3 content of fats. They showed that endogenous rat fat conforms to random distribution, while the exogenous fat is distributed according to an even type of distribution. In chicks, however, both types of fat contained more GS_3 than calculated for random distribution. Although these results are based on fats of low (0.7 to 6.0 per cent) GS_3 content, which makes reliable information difficult to obtain, nevertheless, in the case of the chicks, using triplicated experiments, closely concordant values were found.

The Hilditch principle of even distribution represents a great advance in our knowledge of glyceride structure and doubtless is a useful guide as to the mode of distribution of fatty acids in natural glycerides. It was not intended, however, as a precise mathematical description (1). Moreover, the experimental techniques for glyceride structure determination are not yet sufficiently refined to attempt a very exact description of glyceride types. Until this problem has been solved, arguments as to the relative merits of various theories of glyceride structure are unlikely to lead to a final conclusion.

Kartha (147) has reported that progressive dilution of GS_3 with unsaturated glycerides produces an even series of decrements of the melting point. This has been confirmed by Brooker & West (148) who found, however, contrary to Kartha, that the melting point decrement of the mixture was not independent of the nature of the non-GS_3.

Autoxidation

Initiated by Farmer (149) in 1942, the concept of the mechanism of the autoxidation of fat which involves the participation of the hydrogen in the α-methylenic group adjacent to the double bond, in association with a

resonating free-radical-chain mechanism, has continued to receive experimental support. Along with other evidence, iodometric and polarographic studies have confirmed that monomeric monohydroperoxides are the main primary products of autoxidation of the methyl esters of oleic (150), linoleic (151), and linolenic (152) acids.

From consideration of resonance energies Bolland (153) postulated that in the autoxidation of linoleic acid, of the three possible monohydroperoxides, the conjugated 9- or 13-hydroperoxide would be formed in preference to the nonconjugated 11-hydroperoxide. This was confirmed experimentally by Bergstrom (154), and subsequently by Khan, Lundberg & Holman (155) using methyl linoleate autoxidised under various conditions. However, in photochlorophyll-oxidised methyl linoleate, evidence for the formation of some 11-hydroperoxide was obtained. Khan (156) showed, in addition, that methyl linolenate behaved similarly on autoxidation, giving conjugated monohydroperoxides, but on photochlorophyll oxidation some unconjugated 11- and 14-hydroperoxides were also found. It is believed (155) that photochlorophyll oxidation proceeds differently from autoxidation and is not a chain reaction.

The naturally-occurring unsaturated fatty acids are almost invariably of the *cis* configuration. During autoxidation *cis-trans* isomerisation of the double bond occurs to a considerable and variable extent, depending on conditions. The possibility of the addition and removal of oxygen, to and from the double bond, giving rise to the formation of *trans-* isomers in the unoxidised unsaturated esters (157), is counterindicated, as the unoxidised fractions from autoxidised methyl linolenate (152) or methyl oleate (158) are not measurably isomerised to the *trans* forms. Hence it is evident that the conversion of *cis* to *trans* double bonds and peroxide formation occur in the same molecule, and from the nature of autoxidation of methyl linoleate the isomerisation of the second double bond is independent of the peroxide-forming reaction.

Although the initial stages of autoxidation have been considerably clarified, much remains to be understood about the nature of the subsequent reactions. Coleman, Knight & Swern (159), however, have demonstrated that, during the course of autoxidation in the dark at 80°C., methyl oleate formed essentially only monohydroperoxide up to the level of 15 per cent peroxide. Thereafter, up to the peak value of 35 to 40 per cent, hydroxy, oxirane, and carbonyl compounds were also formed, while beyond this peak value the mixture was first completely converted to oxygenated compounds with only one functional group in the chain, before multiple attack by oxygen or peroxide occurred.

Complex Lipides

Lea and co-workers (160, 161) have shown, using silica impregnated paper and 20 to 30 per cent v/v methanol in chloroform that glycerophospholipide components may be separated. Typical R_F values were as follows: phosphatidylethanolamine 0.85, lecithin and ethanolamine plasmalogen 0.65, lysophosphatidylethanolamine and sphingomyelin 0.5, and lysolecithin 0.3.

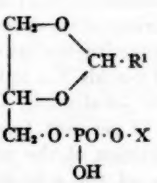
Although lecithin and plasmalogen frequently overlapped, as did also sphingomyelin and lysophosphatidylethanolamine, the individual constituents were distinguishable by use of appropriate reagents. No appreciable separation according to degree of unsaturation or chain length of the fatty acids occurred. Dieckert & Reiser (162) used glass fiber filter paper impregnated with silicic acid which permitted the location of the separated components by spraying with 20 per cent H_2SO_4 and heating.

Collins & Wheeldon (163) have opened up new possibilities for the separation of phospholipide mixtures by conversion of the free amino groups to nitrophenyl derivatives with fluoro-2, 4-dinitrobenzene. This was followed by esterification of the secondary phosphates and the carboxyl group of serine with diazomethane. Separation of rat liver lipides treated by this procedure, using "Hyflo-super-cel" columns, revealed some unknown constituents, in addition to phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, small amounts of sphingomyelin, and inositol phospholipides. However, the use of diazomethane in connection with phospholipide work demands a note of caution. Baer & Maurukas (164) have shown that this reagent cleaves the ester linkage in phosphatidyl serine and phosphatidylethanolamine, leaving intact the nitrogenous base and the optically pure phosphatidic acid dimethyl esters.

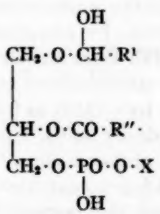
From the results obtained with a series of amino-containing phospholipides Lea & Rhodes (165) concluded that the ninhydrin colour reaction could be used for the quantitative estimation of mixed amino-containing phospholipides in lipide extracts.

Recently isolated phospholipide constituents include an ethanol-insoluble phospholipide present in cat and rabbit livers (166) and with properties similar to the inositol monophosphate lipide isolated from heart muscle by Faure & Morelec-Coulon (167). In addition, Klenk & Dohmen (168) purified a phosphatidylethanolamine fraction from cow liver.

Klenk & Debuch (169) have shown that formula I, originally given by Feulgen & Bersin (170) to plasmalogens, does not apply to the naturally occurring constituents but only to compounds which have been modified by the action of alkali used in the course of their isolation. The naturally occurring plasmalogens, according to Klenk & Debuch (169), are represented by formula II.



I



II

R = an alkyl group and X = choline or ethanolamine

In accordance with this suggestion it was shown that a hydrogenated plasmal-rich lecithin fraction, on hydrolysis with methanolic HCl, gave chimyl and batyl α-phosphoric acid, while acetic acid at 37°C. produced fatty aldehyde and lysolecithin.

Kiss, Fodor & Banfi (171) have further established the D-erythro structure of sphingosine by conversion of the ozonolysis product to 2-amino-3, 4-dihydroxybutyric acid of known configuration, while Shapiro & Segal (172) synthesised *trans*-DL-erythro-1,3-dihydroxy-2-amino-4-octadecene starting from ethyl α(*trans*-2-hexadecenoyl)acetoacetate obtained from the acid chloride and ethyl acetoacetate. Conversion to the α-acetamido ester followed by reduction of the β-oxogroup yielded two diastereoisomeric carbinols which were separated by crystallization. One of these, on saponification and reduction, yielded the desired 1,3-dihydroxy-2-amino-4-octadecene, the erythro configuration of which was proved by its hydrogenation to the corresponding dihydrosphinogosine and the *trans* structure from its characteristic absorption at 10.3μ.

A reinvestigation by Klenk & Faillard (173) of the optically active acid obtained by the ozonolysis of sphingosine showed that the acid was not α-amino β-γ-, as formerly supposed (174), but β-amino α-γ-dihydroxybutyric acid. Oxidation of the N-acetyl derivative gave L-serine identical with the naturally-occurring acid. Zabin & Mead (175) considered that the evidence from feeding labelled compounds to rats showed that sphingosine originates from a C_{16} fatty acid-like intermediate and the α and β carbon atoms of serine.

The cerebroside sulphuric ester isolated by Blix (176) from beef brain was shown to contain cerebronic acid, sphingosine, and galactose, but the position of the sulfate group was unknown. This group has now been shown by Thannhauser, Fellig & Schmidt (177) to occupy the primary hydroxyl of carbon atom 6 of the galactose.

Waxes

Murray & Schoenfeld have continued their studies on wax constituents. In wool wax they established by urea fractionation and amplified distillation of the acetates that the even series of *n*-alcohols from C_{18} to C_{30} is present and form about 1 per cent of the wax (178). From carnauba wax by chromatography of the unsaponifiable matter on alumina they separated a diol fraction which, by amplified distillation of the acetates, has been further resolved (179). With similar techniques the ω-hydroxy acids were also separated and a quantitative picture of carnauba wax constituents has been given for the first time (180) as follows: even-numbered *n*-acids, C_{18} to C_{30}, 18 per cent; -ω-hydroxy acids, C_{18} to C_{30}, 27 per cent; -*n*-alcohols, C_{24} to C_{34}, 51 per cent, and -α-ω-diols, C_{22} to C_{28} and higher, 3.5 per cent, together with less than 0.5 per cent hydrocarbons. The recognition of the presence of α-ω-diols and the more definite characterisation of the ω-hydroxy acids

represents a considerable achievement in the separation of complex mixtures and may well explain some of the outstanding and desirable physical properties of the wax.

Hougen (181) isolated from human sebum the even-numbered saturated alcohols, C_{14} to C_{24}, and the *n*-unsaturated alcohols, eicos-10-enol, docos-12-enol, and tetracos-14-enol as well as the C_{20}, C_{22}, and C_{24} *iso* alcohols. It was previously shown by Weitkamp, Smiljanic & Rothman (182) that acids of human sebum consisted of *n*-odd and even-numbered saturated and unsaturated C_7 to C_{24} acids. The absence of correlation between the structure of the acids and alcohols in hair wax stands in striking contrast to wool wax where these constituents both belong to the same series of normal, *iso*, *anteiso*, and α-hydroxy compounds (183). However, the absence of *n*-odd-numbered alcohols in human sebum is contradicted by Brown, Young & Nicolaides (184) who, by use of mass spectrometry, found *n*-alcohols within the range C_{16} to C_{27}, the even-numbered constituents predominating.

Chibnall & Piper (185), from a survey of the constituents of plant and insect waxes, considered that all the saturated, ketonic, and hydroxy acids, as well as the primary alcohols, contained an even-number of carbon atoms, whereas the paraffins, ketones, and secondary alcohols which are probably derived from these acids by decarboxylation contain an odd-number of carbon atoms. Rose petal wax, however, appeared to be anomalous as Prophète (186) had shown a range of odd- and even-numbered paraffins between C_{16} and C_{35}. Chibnall, El Mangouri & Piper (187) therefore re-examined this wax and established that only odd-numbered paraffins from C_{21} to C_{35} were present. The picture provided by Chibnall and co-workers of the composition of plant and insect waxes, however, while perhaps correct in broad outline, may not be true in regard to the minor constituents, as Wanless, King & Ritter (188) found in pyrethrum wax all of the normal odd- and even-numbered hydrocarbons from C_{24} to C_{36}, the presence of which was confirmed by mass-spectrometer analysis.

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METABOLISM OF PURINES AND PYRIMIDINES[1,2]

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The emphasis of recent reviews on structural aspects of nucleic acid chemistry and the substantial achievement represented by the publications of the past year on the metabolism of purine and pyrimidine nucleotides has led to the selection of enzymic reactions operative in the synthesis of these compounds as the principal subject of this review. Within the year an excellent and encyclopedic reference on nucleic acids, edited by Chargaff & Davidson (1), has become available; in this, chapters by Brown, Reichard, and Schlenk, respectively, develop the background and current status of the several aspects of nucleotide and nucleic acid metabolism.

The isolation of 5-phosphoribosylpyrophosphate (PRPP) by Kornberg, Lieberman & Simms (2, 3) and the demonstration of its pivotal role in the biosynthesis of purine and pyrimidine nucleotides underlies much of the current success in delineation of synthetic pathways. This ester is formed enzymically in several tissues through the reaction of ribose-5-phosphate and adenosinetriphosphate by a pyrophosphorylation of carbon-1 of ribose, and the formation of adenosinemonophosphate (AMP).

$$\text{ATP} + \text{R-5-P} \rightarrow \text{PRPP} + \text{AMP} \qquad 1.$$

A purification of the ribosepyrophosphorylase of pigeon liver acetone powder was achieved and Mg^{++} was found essential for activity. Like ribose-1-phosphate, PRPP is labile to heat and mild acid, decomposing completely at 65°C. to ribose-5-phosphate and pyrophosphate at pH 4.0 in 40 min., or at pH 3.1 in 15 min. In PRPP isolated and purified by ion exchange chromatography, the molar ratio of pentose:acid labile P:total P is 1:2:3. The configuration of PRPP at C-1 has not been determined. However, Wright & Khorana (4) have compared ribose-1-phosphate derived from guanosine by the nucleoside phosphorylase reaction (5, 6) with synthetic ribose-1-phosphate which they prepared from 2,3,5-tri-0-benzoyl-D-ribofuranosyl-1-bromide and triethylammonium dibenzylphosphate and have concluded that the

[1] The survey of the literature pertaining to this review was completed in November, 1955.

[2] The following abbreviations are used: ADP for adenosinediphosphate; AMP for adenosine-5′-phosphate; ATP for adenosinetriphosphate; CDP for cytidine diphosphate; CMP for cytidine-5′-phosphate; CoA for coenzyme A; CTP for cytidine triphosphate; DNA for deoxyribonucleic acid; DPN for diphosphopyridine nucleotide; GDP for guanosine diphosphate; GMP for guanylic acid; GTP for guanosine triphosphate; IDP for inosine diphosphate; IMP for inosine-5′-phosphate; PRPP for 5-phosphoribosylpyrophosphate; RNA for ribonucleic acid; UDP for uridine diphosphate; UMP for uridine-5′-phosphate; UTP for uridine triphosphate.

synthetic ester is β-D-ribofuranose-1-phosphate. This assignment of structure is based upon the chromatographically identical behavior of the differently derived ribose-1-phosphates, the failure of synthetic ribose-1-phosphate to participate in the nucleoside phosphorylase reaction, and the demonstration that only the natural ribose-1-phosphate reacts with dicyclohexylcarbodiimide (7) as a phosphate ester bearing a *cis*-hydroxyl function to form a cyclic phosphate and an N-phosphoryl urea. Therefore, the ribose-1-phosphate derived from guanosine, a compound known to have a β-configuration, is believed to be an α-D-ribofuranose-1-phosphate, and it is probable that extension of these studies will demonstrate this configuration for PRPP.

PRPP functions in nucleotide synthesis through transfer of the 5-phosphoribosyl group to a nitrogen atom of an aglycone; this reaction is catalyzed specifically by a group of pyrophosphorylases which will be described below.

Pyrimidine Biosynthesis

Evidence presently available supports the sequence of reactions described below as the major device for achieving net increments of pyrimidine nucleotides *de novo*. However, under certain circumstances such as in mutant organisms, neoplastic tumors, or as a consequence of resistance to antimetabolites, alternate pathways of pyrimidine metabolism may assume greater importance. Some of these features have been considered in a recent review by Welch (7a).

Synthesis of carbamyl-L-aspartic acid (L-ureidosuccinic acid).—Clarification of the mechanism of carbamyl group transfer reactions was achieved by Jones, Spector & Lipmann (8) with the isolation of carbamylphosphate from bacterial extracts which catalyze the reaction:

$$CO_2 + NH_3 + ATP \rightarrow NH_2\text{—}\overset{\overset{\displaystyle O}{\|}}{C}\text{—}OPO_3^{=} + ADP \qquad 2.$$

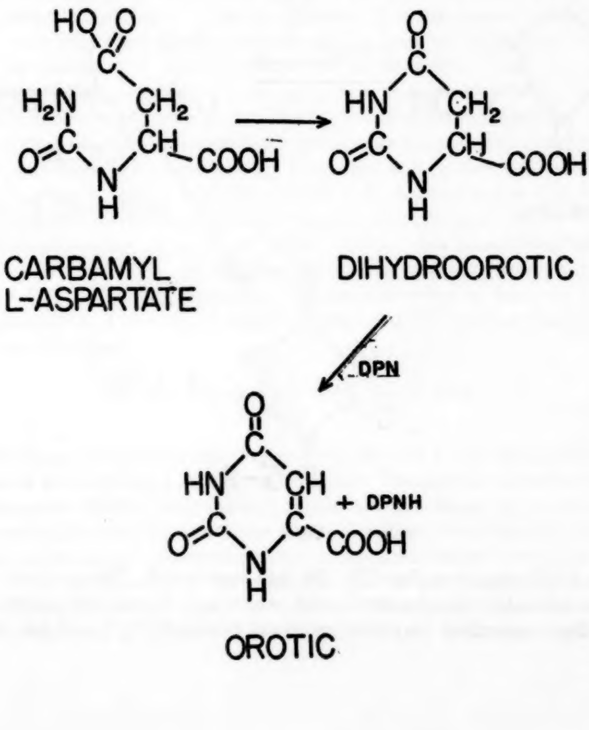
Carbamylphosphate also was synthesized by reacting KCNO with inorganic phosphate and was isolated as the lithium salt. In suitable enzyme systems the natural and synthetic products donate the carbamyl group to the δ-amino of ornithine to yield citrulline (8, 11, 12), or to the α-amino group of aspartic acid to form carbamylaspartate (8, 9, 11). In mammalian enzyme systems N-acylglutamate or carbamylglutamate serves a catalytic function necessary to the formation of carbamylphosphate, as shown by Grisolia & Cohen and their co-workers (10, 11). The "compound X" of Grisolia & Cohen (10), originally identified as a glutamic acid derivative, is now considered by Marshall, Hall & Cohen (11) to be identical with carbamylphosphate while Grisolia *et al.* (12), on the basis of differential decomposition rates, hold a dissenting opinion which favors separate identities of the two compounds. The latter view is supported by Reichard, Smith & Hanshoff (9), who found two carbamyl donor compounds generated in a liver particulate extract in the presence of acetylglutamate, radioactive CO_2, ATP, and Mg^{++}. One of the compounds was identified as a carbamylphosphate, the other has the properties of a compound in which carbamylphosphate is bound to acetylglutamate

and is designated "compound X." In this system citrulline gave rise to "compound X" and carbamylphosphate and served thereby as a carbamyl donor in the synthesis of carbamylaspartate. It is clear that the carbamyl group of citrulline may be incorporated into pyrimidines through carbamylaspartate, as the work of Schulman & Badger (13), Smith & Stetten (14), and Heinrich, Dewey & Kidder (15) indicated. The failure of incorporation of the carbamyl group of citrulline into pyrimidine, reported earlier by Reichard (16) and more recently by Cooper, Wu & Wilson (17) for rat liver slice systems [see also (13, 14, 15)], is probably attributable to the preferential, if not exclusive, utilization of citrulline in the urea cycle. The following over-all reactions for the synthesis of carbamylaspartate in mammals must remain ambiguous until the role and identity of "compound X" is determined.

$$\begin{array}{c}\text{Citrulline} + (\text{ADP} - \text{ATP}) \\ \text{or} \\ CO_2 + NH_3 + \text{ATP}\end{array} \xrightarrow{\text{acetylglutamate}} (\text{"Cpd X"}) \rightarrow NH_2\text{—CO—}OPO_3^{=} + \text{ADP} \qquad 3.$$

$$NH_2\text{—CO—}OPO_3^{=} + \text{L-Aspartate} \rightarrow \text{Carbamyl-L-aspartate} + HPO_4^{=} \qquad 4.$$

Enzymic conversion of carbamylaspartate to orotic acid.—The precursor relationship of carbamylaspartate to orotic acid and nucleic acid pyrimidines was established by nutritional experiments in microorganisms and isotope



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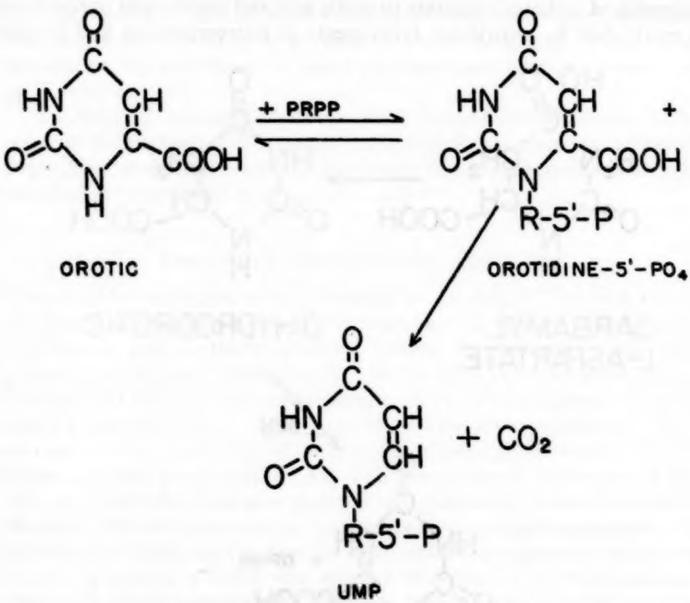
experiments in microorganisms and mammals [see Reichard in (1)]. The enzymic conversion of carbamylaspartate to orotic acid in a purified system was demonstrated by Lieberman & Kornberg (18) who isolated two enzymic activities from a soil bacillus, one of which effects ring closure of carbamylaspartate to dihydroorotic acid and the other, a DPN-dependent dehydrogenase, removes two hydrogen atoms at C-5, 6 of dihydroorotic to yield orotic acid. It is of interest that the first authentic specimen of dihydroorotic acid was obtained through the use of this system. That similar reactions are operative in mammals was indicated by the work of Cooper, Wu & Wilson (17) (see equation 5).

Synthesis of uridine nucleotide from orotic acid.—For several years the role of orotic acid in the biosynthesis of pyrimidine nucleotides had posed the refractory problem of the mechanism of conversion of the aglycone to nucleotide linkage. In fact, it was the investigation of this problem that led Kornberg, Lieberman & Simms (2) to the discovery of PRPP and the nucleotide pyrophosphorylase reactions. From autolysates of yeast, the most active of several natural sources examined, they purified orotidylic pyrophosphorylase



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and orotidylic decarboxylase (19, 20) (see equation 6). The purified nucleotide pyrophosphorylase for orotic acid, which may be assayed spectrophotometrically by coupling the above reactions, required Mg^{++} and was specific;

uracil, cytosine, and dihydroorotic acid were not reactants. It is noteworthy that so far only orotic acid has been found to participate in pyrimidine nucleotide pyrophosphorylase reactions. In the absence of orotidylic decarboxylase, orotidine-5′-phosphate accumulated and was isolated by ion exchange chromatography and characterized. Enzymic decarboxylation of orotidylic acid was found to be irreversible.

Synthesis of cytidine nucleotide.—The conversion of uridine nucleotide to cytidine nucleotide was found by Lieberman (21) to take place at the triphosphate level in extracts of *Escherichia coli*, by a reaction dependent upon ammonia and ATP.

$$UTP + NH_3 + ATP \rightarrow CTP + ADP + P \qquad 7.$$

Glutamine and asparagine, as well as glutamic and aspartic acids, failed to react; and the same relative isotope content was found in $N^{15}H_3$ and in the amino group of the CTP formed in the reaction mixture. It is of interest that inosine triphosphate was not aminated in this system; also, it could not replace the requirement for ATP. The amination of uracil as the free base, the nucleoside, or the mononucleotide was not achieved. Although both ADP and UDP participated in CTP synthesis, they were less efficient and probably were utilized through intermediation of phosphotransferase reactions.

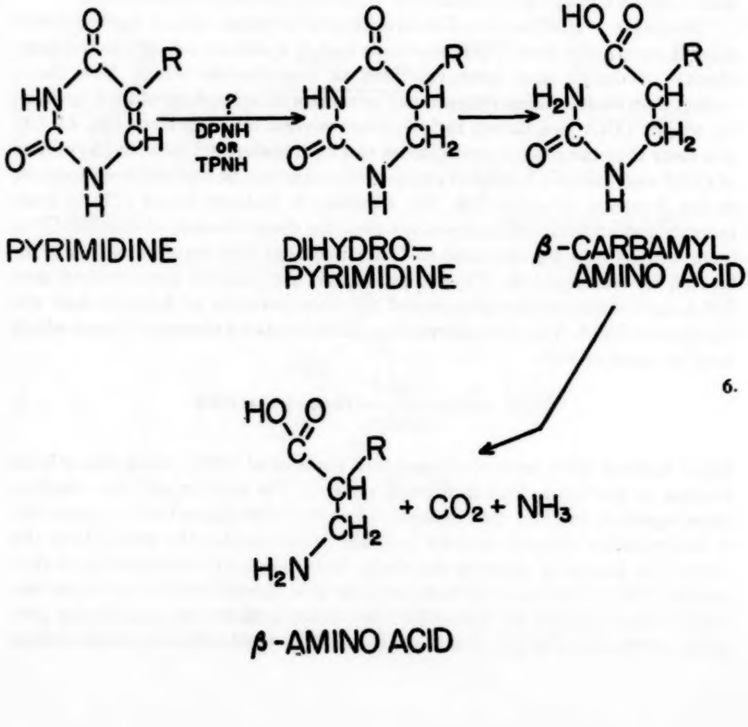
Biosynthesis of thymine.—The synthesis of thymine cannot easily be considered separately from DNA synthesis and is therefore subject to the complexity of design and interpretations of experiments which have been undertaken to determine renewal and precursor relationships of high molecular weight DNA [see Brown in (1)]. From several investigations (22, 23, 24) it is clear that the ribosyl pyrimidines can be transformed into the thymidine of DNA and that the 5-methyl group of thymine can be derived from formate or the β-carbon of serine (25, 26). Friedkin & Roberts found (27) in bone marrow and embryo cell suspensions that the deoxyriboside of uracil-2-C^{14} is incorporated into the thymine of DNA and that this transformation is inhibited by Aminopterin. Thymidine diluted the activity incorporated into DNA, and deoxyuridine augmented the incorporation of formate into the thymine of DNA. The over-all reaction must involve a number of steps which may be summarized:

$$\text{Deoxyuridine} \xrightarrow[\text{Formate}]{\text{Co—F}} \text{Thymidine} \rightarrow \text{DNA} \qquad 8.$$

These findings have been confirmed by Prusoff *et al.* (27a), using either bone marrow or Ehrlich ascites tumor cell systems. The specific activity relationships reported, the fact that deoxycytidine does not dilute the incorporation of deoxyuridine activity, and the isolation of radioactive thymidine from the incubation mixtures, support the direct formylation of deoxyuridine to thymidine. The occurrence of deoxyuridine and deoxycytidine in tissue extracts, demonstrated by Schneider (28), lends indirect support to the proposed mechanisms of thymidine synthesis. The metabolic interrelationships

of thymidylic, 5-methyl deoxycytidylic, and 5-hydroxymethyldeoxycytidylic acid are not known. The investigation of Cohen & Weed (29) showed that the hydroxymethyl group of bacteriophage hydroxymethyl cytosine was not derived from the methyl group of host DNA-thymine, and that host cytosine but not thymine contributed to the formation of virus hydroxymethylcytosine.

Pathways of pyrimidine metabolism which have not been shown to yield net synthesis of pyrimidine.—This cumbersome title has been adopted to avoid inferences implicit in the terms "degradative" or "catabolic" pathways. In distinction to those isolated reactions previously discussed, which may lead to absolute increases in pyrimidine nucleotide from acyclic precursors, the enzymic conversions considered here involve transformation of preformed pyrimidine and the apparent equilibrium of these reactions is toward formation of acyclic products. The observations made by Rutman *et al.* (30) and Holmes *et al.* (31) that C-2 labeled uracil and thymine are extensively degraded in mammals and in liver slices to yield radioactive CO_2 indicated an active mechanism for disruption of the ureido portion of the pyrimidine ring. The course of the over-all reaction was indicated by Fink *et al.* (32, 32a)



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through chromatographic isolation from the urine of the products derived from uracil and thymine, and from *in vitro* experiments with a rat liver enzyme preparation. When uracil was administered to rats, Fink *et al.* (32, 32a) isolated from urine β-aminopropionic acid and β-carbamylaminopropionic acid; in similar experiments with labeled thymine, β-carbamylaminoisobutyric acid and β-aminoisobutyric acid were identified (32a, 33). The scope and importance of these reactions have been extended by several observations. In rat liver slice incubation mixtures containing high substrate concentrations of uracil-2C^{14}, Canellakis found (34) a considerable proportion of the labeled uracil converted to pyrimidine nucleotides and nucleic acid pyrimidine, and, in confirmation of the earlier observation of Rutman *et al.* (30), a much smaller proportion of the labeled uracil was degraded to CO_2 than when low substrate concentrations of uracil were employed. The incorporation of labeled uracil into the nucleic acids of intestinal epithelium of the rat was reported by Leibman & Heidelberger (34a), and the work of Lagerkvist & Reichard (34b) demonstrated that in the mouse uracil was as efficiently utilized for synthesis of nucleic acid pyrimidines as orotic acid in both visceral tissues and Ehrlich ascites cells. Whether the extent of uracil degradation in tissues limits the availability of the compound for pyrimidine nucleotide biosynthesis, as these observations might indicate, remains to be determined. Also it would be of considerable importance to know, under suitable conditions in normal tissues or in tumors where uracil utilization was found by Rutman *et al.* (35), whether carbamyl β-alanine might be incorporated into pyrimidine nucleotides and nucleic acid pyrimidine.

The general question of the significance of dihydropyrimidine compounds in biosynthetic mechanisms is raised by the demonstrated metabolic role of dihydroorotic acid in pyrimidine synthesis. Also, interest in these compounds is emphasized by the finding of Grossman & Visser (36) that C^{14}-labeled cytidine was converted in rat liver slices to dihydrocytidylic acid of nearly identical specific activity and the report of Herbert *et al.* (37) that dihydrouridine 5′-phosphate is converted to the diphosphate and triphosphate in liver homogenates. Lieberman, Kornberg & Simms (19) found that orotic acid nucleotide phosphorylase will not react with dihydroorotic acid, and presumably this restricts the role of the dihydro compound to the prenucleotide level in what appears to be the pathway of biosynthesis *de novo* of pyrimidine nucleotides. However, transformations of pyrimidine nucleotides through saturated compounds may have an importance not yet demonstrated.

Pyrimidine bases may be converted to nucleotides through operation of several mechanisms [see Schlenk in (1)]. In mammals, the nucleoside phosphorylase (6) and nucleoside kinase (38 to 41) or phosphotransferase reactions (42) could operate sequentially to achieve a synthesis of pyrimidine nucleotide from pyrimidine, ribose-1-phosphate, and ATP. In microorganisms these reactions and the transnucleosidation reactions described by McNutt (43) are undoubtedly responsible in part for the efficient utilization

of pyrimidine bases for nucleic acid and nucleotide synthesis. That these mechanisms normally are not operative in mammals is attested by the extremely low incorporation of labeled pyrimidines into tissue nucleic acids [see Brown in (1)]. In tumors, or in the case of resistant strains emerging consequent to the presence of an antimetabolite, these reactions may assume considerable importance.

Biosynthesis of Purines

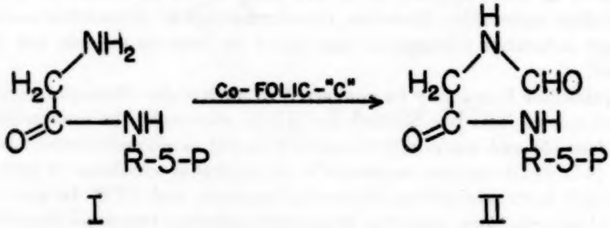
The synthesis of aminoimidazole ribosylphosphate.—The achievement of Goldthwaite, Peabody & Greenberg (44, 45, 48) and of Buchanan and his co-workers (46, 49, 50) has delineated the enzymic reactions whereby glycine is assimilated into an imidazole ribosylphosphate. That glycinamide ribosylphosphate (1) is an intermediate in the sequence was established by Goldthwaite *et al.* (44, 45) and confirmed by Hartman, Levenberg & Buchanan (46). However, it had been shown by Buchanan *et al.* (47) that glycinamide was not an efficiently utilized purine precursor. Recently Goldthwaite *et al.* (48) found that synthetic 5-phosphoribosylamine can replace the requirement for PRPP and glutamine in a pigeon liver enzyme system which synthesizes glycinamide ribosylphosphate (I) and suggested the following sequence of reactions:

$$\text{PRPP} + \text{Glutamine} \rightarrow \text{H}_2\text{N—R—5P} + \text{PP} \qquad 10.$$

$$\text{H}_2\text{N—R—5P} + \text{Glycine} \rightarrow \text{H}_2\text{N—CH}_2\text{—CO—NH—R—5P} \qquad 11.$$

These reactions are in striking contrast to the synthesis of heterocyclic ribosyl structures in the formation of pyrimidines. Although the product of the enzymic reaction of glutamine and PRPP has not been isolated and characterized, the efficient utilization of 5-phosphoribosylamine in the subsequent reaction constitutes strong evidence in favor of the validity of this scheme.

The formylation of the amino group of glycinamide ribosylphosphate, a reaction dependent upon a coenzyme derived from folic acid, was demonstrated in purified pigeon liver enzyme systems by Goldthwaite, Peabody & Greenberg (44) to result in the synthesis of formyl glycinamide ribosylphosphate (II). Both (I) and (II) were isolated as isomeric pairs by ion exchange



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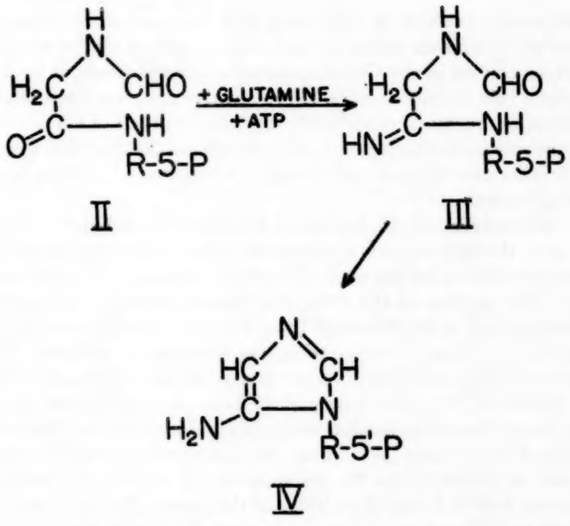
chromatography, but the chemical basis and metabolic significance of this isomerism remains to be determined. Hartman, Levenberg & Buchanan (46) independently isolated the glycinamide ribosylphosphate compounds from purified enzyme reactions and made the important observation that these compounds accumulated in reaction mixtures which contained the antimetabolite azaserine. An explanation of the inhibitory effects of azaserine in purine biosynthesis advanced by these workers (46) follows from their demonstration that glutamine reacts with formylglycinamide ribotide (II) and ATP in a purified pigeon liver enzyme system to yield 5-aminoimidazole ribosylphosphate (IV), through the intermediate formation of the amidine compound (III) (49, 50). It is this sequence of reactions which azaserine blocks, presumably as a glutamine antimetabolite, thus causing an accumulation of the glycinamide ribotides.



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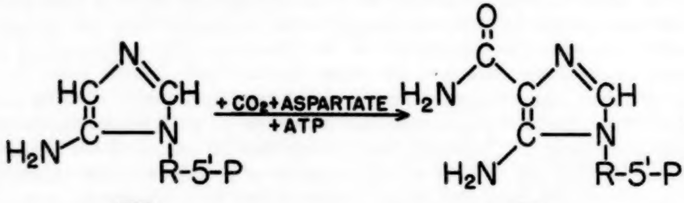
The role of aminoimidazole ribosylphosphate in purine biosynthesis was previously indicated by the work of Love & Gots (51) who demonstrated the accumulation in cultures of an *E. coli* mutant of an aminoimidazole riboside, which was chemically distinct from 5-amino-4-imidazolecarboxamide but which could be transformed into the latter compound in a genetically different mutant of *E. coli*. Evidence for the existence of such an intermediate could also be adduced from the studies of Chamberlain & Rainbow (52) on a biotin-deficient yeast. Although the mechanism and intermediates in the enzymic conversion of (IV) to 5-amino-4-imidazolecarboxamide ribosylphos-

phate (V) are not yet clearly delineated, the Buchanan group (49, 50) reported that aspartic acid, CO_2, and ATP are requirements and that the nitrogen of the amino group of aspartic acid becomes the amide nitrogen.



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Goldthwaite, Peabody & Greenberg (45) have presented evidence which is interpreted to exclude succinate and other members of the Krebs cycle as direct intermediates in the CO_2 incorporation reaction, except for a possible intermediate role of oxaloacetate. A provocative note has been injected into the problem by Shemin's report (53) that the α-carbon of δ-aminolaevulinic acid (in the succinate-glycine cycle this represents the 2-carbon of glycine) is efficiently incorporated into the C-2 and C-8 positions of purines and into the C-6 position as well.

The interconversion of 5-amino-4-imidazolecarboxamide ribotide and inosinic acid through transformylation reactions dependent upon folic acid coenzyme was shown by the work of Flaks & Buchanan (47, 54) and Greenberg (55). The position of the latter reactions in adenylic and guanylic acid biosynthesis is not clear. Although inosinic acid is the first complete purine ring structure resulting from the foregoing sequence of reactions (56), there is reason to question its immediate precursor role in adenylic acid biosynthesis [see Brown in (1)]. It has frequently been suggested that inosinic acid occupies an ancillary role peculiar to the uricotelic organisms. The early work of Kalckar & Rittenberg (57), which demonstrated an extensive incorporation *in vivo* of $N^{15}H_4Cl$ into the amino group of muscle ATP and very low incorporation into N-1 and -3 positions of the purine ring, has generally been interpreted to indicate an active mechanism for the amination of inosinic acid through transfer of amide nitrogen (58). However, results of a recent investigation by Roll & Weliky (59) showed equal labeling in the nitrogen of muscle ATP after feeding N^{15}-adenine equally labeled in positions 1 and 3 and in the amino group. This finding does not support an active reamination mechanism in adenine metabolism and cannot be easily reconciled with conclusions drawn from the earlier investigation (57, 58).

With the demonstration of the adenylosuccinase reaction by Carter & Cohen (60) an intermediate, adenylosuccinic acid (VI) (AMPS), of possible consequence in the biosynthesis of adenylic acid, became available. This reaction, which is analogous to the aspartase reaction and to the enzymic

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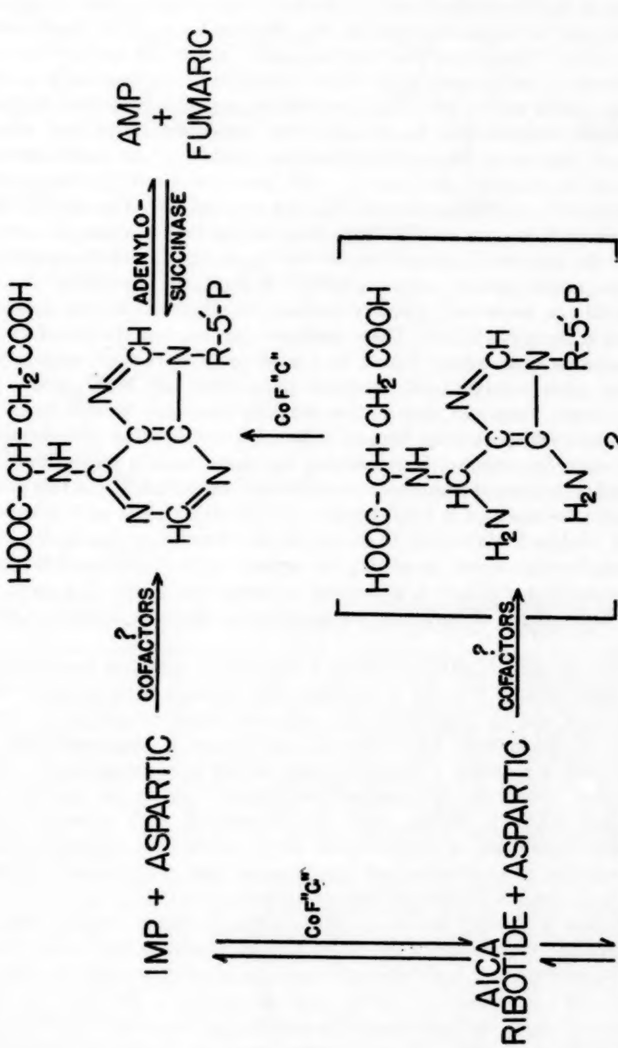
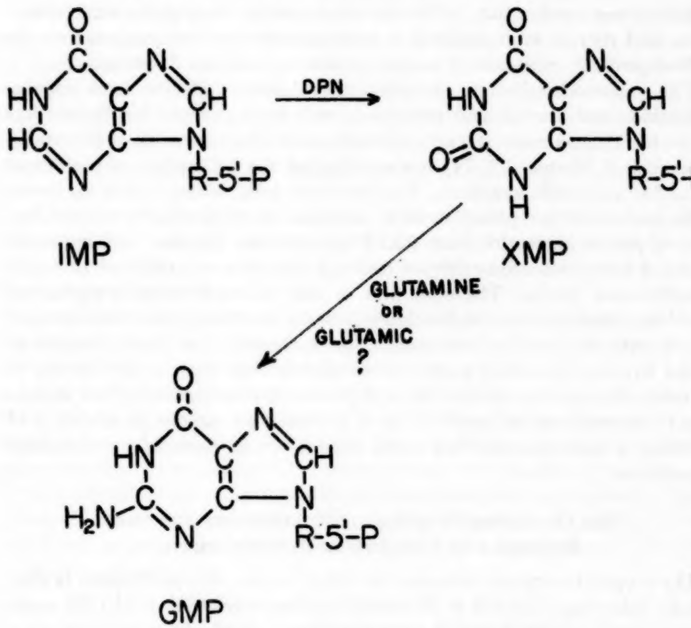

IMP + ASPARTIC
? COFACTORS
VI
HOOC-CH-CH2-COOH
ADENYLO-
SUCCINASE
AMP
+
FUMARIC
CoF"C"
AICA RIBOTIDE + ASPARTIC
PRECURSORS
R-5'-P
?

condensation of arginine and fumaric acid to form argininosuccinic acid (61), led to the hypothesis that adenylosuccinic acid (VI) might occupy a position in the biosynthetic path of adenylic acid similar to that of argininosuccinic acid in arginine synthesis (61). On the basis of the incorporation of formate-C^{14} by pigeon liver homogenates, which led under certain circumstances to the appearance of higher specific activity in adenylosuccinate than in inosinic acid, Carter & Cohen (62) suggested that inosinic acid is not an obligate intermediate in adenylic acid biosynthesis and that adenylosuccinate may arise from a condensation product of an aminoimidazolecarboxamide nucleotide and aspartic acid; a reaction similar to the condensation of citrulline and aspartic acid (61) (see equation 18). The validity of this proposed path has not yet been supported by the isolation and characterization of the required intermediates or the demonstration of its operation in suitable purine mutant microorganisms. Indeed, the possibility that inosinic acid can serve as a primary reactant is suggested by the findings of Abrams & Bentley (63, 64). These workers reported that the transformation of inosinic acid to adenylic acid in a bone marrow enzyme system is dependent upon aspartic acid, inorganic phosphate, K^+, Mg^{++}, and 3-phosphoglycerate. Intermediates in this reaction were not identified, but the results were interpreted by Abrams & Bentley as indicating an intermediary role of adenylosuccinate. In a complex enzyme system, a sequentially prior imidazole analogue of adenylosuccinate cannot be excluded, and this possibility might be assessed if IMP labeled in C-2 rather than in C-8 were employed. Wahba & Shive (65), in studies on the effect of purines and 5-amino-4-imidazolecarboxamide in sparing the aspartic acid requirement for growth in *Lactobacillus arabinosus*, presented evidence for a role of aspartic acid in the biosynthesis of purines at a stage prior to the incorporation of the C-2 carbon.

Guanylic acid (GMP) synthesis from inosinic acid has been independently described by Abrams & Bentley (63, 64) for a bone marrow enzyme preparation and by Lagerkvist (66), who employed extracts of an acetone powder of pigeon liver. These workers and Gehrig & Magasanik (67) have shown that a requisite preliminary step in the transformation is a DPN-dependent oxidation of inosine-5′-phosphate (IMP) to xanthosine-5′-phosphate (XMP). Earlier work by Magasanik with a mutant microorganism exhibiting a requirement for guanine had demonstrated the accumulation of xanthosine and clearly implicated xanthosine-5′-phosphate as an intermediate in the synthesis of guanylic acid (68).

Abrams & Bentley (63) found that ATP and glutamic acid or glutamine were specific requirements for guanylic acid synthesis and suggested as a possible intermediate a glutaric acid analogue of adenylosuccinate. However, Lagerkvist's experiments indicate that glutamine is the donor of the 2-amino group and implicate an amide-transfer mechanism in the biosynthesis of guanylic acid (66). The early experiments of Barnes & Schoenheimer (69), employing $N^{15}H_4Cl$ *in vivo*, showed a much higher isotope concentration in the amino group of guanine than in the amino group of adenine. Also,



16.

Reichard's experiments with N^{15}-glycine resulted in a higher isotope content in the amino group of guanine, presumably because of the partial conversion of glycine to ammonia and a more rapid utilization of ammonia for synthesis of the amino group of guanine (70). These studies may be interpreted to mean that fundamentally similar mechanisms are operative at different rates in guanine and adenine nucleotide biosynthesis, or that the method of synthesis of the amino groups is fundamentally different. It is also possible that two paths exist for guanylic acid biosynthesis, one of which involves an aminoimidazole derivative and requires glutamic acid, and the other depending upon an amide-transfer mechanism which converts xanthosinic acid to guanylic acid.

A reaction of great interest was discovered by Alivisatos & Woolley (71) to take place in the presence of a purified spleen enzyme which catalyzed the exchange of 5-amino-4-imidazolecarboxamide with the nicotinamide moiety of DPN yielding the aminoimidazolecarboxamide analogue of DPN. Pyridine analogue syntheses of this type have been demonstrated with tissue diphosphopyridine nucleotidases by Burton, Kaplan & Ciotti (72). Abrams & Bentley (64) reported a requirement for DPN in the synthesis of adenylic acid from inosinic acid in bone marrow enzyme preparations, an observation which might suggest the participation of a reaction of this type in adenylic acid biosynthesis. However, in the dialyzed preparations employed

in their recent studies (63) DPN was not required for adenylic acid biosynthesis, and its role in guanylic acid synthesis now can be attributed to the DPN-dependent oxidation of inosinic acid to xanthosine-5′-phosphate.

The rapid assimilation of isotopically labeled adenine into adenine nucleotides and nucleic acid purines *in vivo* demonstrated by Brown [see Brown in (1)] and more recently and definitively by the work of Kornberg, Lieberman & Simms (73, 74) has established the utilization of preformed purine for nucleotide synthesis. The latter workers, through their discovery of the nucleotide pyrophosphorylase reactions, have demonstrated the synthesis of purine nucleotide from PRPP and adenine, guanine, and hypoxanthine and have presented evidence that separate enzymes catalyze the reactions for each purine. The equilibria of the purine nucleotide pyrophosphorylase reactions favor nucleotide synthesis, but reversibility was demonstrated with radioactive reactants. The extremely low incorporation of labeled hypoxanthine and guanine into nucleic acid purines [see Brown in (1)] raises the question of the role of degradative reactions of purine metabolism in determining the availability of potential precursors for nucleic acid synthesis, a question accorded some importance in pyrimidine nucleotide biosynthesis.

The Coenzyme Function of Nucleotides and the Synthesis of Nucleotide Pyrophosphate

The scope of enzymic reactions in which nucleotides participate is continually enlarging. Kalckar & Klenow's excellent review in 1954 (75) made an important contribution to the literature of this field and should become a standard reference. To the many reactions in which adenosine and uridine nucleotides participate have now been added those of guanosine and cytidine nucleotides. Sanadi, Gibson & Ayengar (76), employing a soluble enzyme preparation which oxidizes α-ketoglutarate, found that in the accompanying esterification of phosphate, guanosine diphosphate or inosine diphosphate, but no other nucleoside diphosphate, was the primary phosphate acceptor. Since then the work of Keller & Zamecnik (77) has extended the coenzyme role of guanosine nucleotides to the enzymically catalyzed incorporation of isotopically labeled amino acid into the microsome protein fraction of a purified liver preparation. Recently a guanosyl transferase reaction similar to the uridyl transferase reactions has been described by Munch-Petersen in the enzymic synthesis and pyrophosphorolysis of guanosine diphosphate mannose (78), a compound previously isolated by Cabib & Leloir (79).

$$\text{GDP mannose} + \text{PP} \leftrightarrows \text{GTP} + \text{Mannose-1-phosphate} \qquad 17.$$

With the discovery of the function of cytidine triphosphate in lecithin biosynthesis by Kennedy & Weiss (80), the first evidence of a coenzyme function of cytosine nucleotide was presented.

$$\text{CTP} + \text{Phosphorylcholine} \rightleftarrows \text{Cytidine diphosphate choline} + \text{PP} \qquad 18.$$

$$\text{Cytidine diphosphate choline} + \text{Acceptor} \rightarrow \text{Lecithin} + \text{CMP} \qquad 19.$$

The participation of uridine nucleotides in enzymic reactions has been extended further by the demonstration of Strominger *et al.* (81) of the enzymic oxidation of uridine diphosphate glucose through a DPN-dependent reaction to uridine diphosphate glucuronic acid. Storey & Dutton (82) isolated the latter compound from liver and presented evidence for its participation in glucuronide synthesis through a glycosyl transfer reaction:

$$\text{UDP Glucuronic acid} + \text{ROH} \rightarrow \text{UDP} + \text{R—O—Glucuronic acid} \qquad 20.$$

The presence of uridine diphosphate galactosamine in liver has been demonstrated by Pontis (83), and the finding of uridyl transferase reactions in the mammary gland has implicated this activity in the synthesis of lactose from glucose-1-phosphate [Smith & Mills (84)]. Strominger's isolation of uridine diphosphate glucosamine phosphate and sulfate is of great potential importance to the development of an understanding of the mechanisms of synthesis of chondroitin esters and mucopolysaccharides (85).

The purification of an enzyme from yeast which catalyzes an exchange of P^{32}-labeled pyrophosphate and ATP with a specific requirement for acetate has been described by Berg (86). From this reaction evidence was obtained for the participation of the intermediate, adenylacetate, in activation of the carboxyl group of acetate through formation of an adenosine acylphosphate. A compound synthesized from disilver adenylate and acetyl chloride exhibited the properties of adenylacetate and was shown to participate in the formation of acetyl CoA.

$$\text{ATP} + \text{Acetate} \rightleftarrows \text{Adenylacetate} + \text{PP} \qquad 21.$$

$$\text{Adenylacetate} + \text{CoA} \rightarrow \text{Acetyl CoA} + \text{AMP} \qquad 22.$$

Berg indicates an extension of this reaction mechanism to amino acid activation on the basis of a methionine-dependent pyrophosphate exchange reaction with ATP also found in yeast. Hoagland (87) observed a P^{32}-pyrophosphate exchange reaction with ATP in a liver enzyme system which was increased threefold by the addition of amino acids and developed a somewhat analogous formulation of amino acid activation as intermediate in the process of peptide bond synthesis. The implications of these findings, which derive from the earlier work of Lipmann and his associates (88, 89) are, of course, very great.

Following the isolation of purine and pyrimidine ribosyl-5′-mono-, di-, and triphosphates (90 to 93) from tissues, several enzymic reactions, in addition to the nucleotidyl transferases, leading to synthesis of nucleotide pyrophosphate were recognized. Potter *et al.* (94) found that inorganic P^{32} rapidly equilibrated with the di- and triphosphates of adenosine, guanosine, uridine, and cytidine in rat liver *in vivo*. Subsequently, Herbert, Potter & Takagi (95), in a detailed study of nucleotide phosphorylation in rat liver fractions, showed that UMP is phosphorylated to UDP by reacting with ATP in the supernatant fraction and that UDP was phosphorylated to UTP in mitochondria. These studies support adenine nucleotides as the primary

acceptor of phosphate during aerobic phosphorylation and as the phosphate donor in the reactions leading to UDP and UTP synthesis. Independently, several laboratories discovered enzymic reactions of the adenylate kinase (myokinase) type, involving ATP and 5′-purine and pyrimidine mononucleotides. Enzymes from yeast which effect transphosphorylations among the nucleotides of uridine, guanosine, and adenosine were purified by Lieberman, Kornberg & Simms (96, 97). Similar reactions in liver enzyme preparations were observed by Strominger, Heppel & Maxwell (98), who extended the reactant pairs to ATP and cytosine-5′-phosphate (CMP) and CTP and AMP.

$$\text{UMP} + \text{ATP} \rightleftharpoons \text{UDP} + \text{ADP} \tag{23.}$$

$$\text{AMP} + \text{UTP} \rightleftharpoons \text{ADP} + \text{UDP} \tag{24.}$$

$$\text{UMP} + \text{UTP} \rightleftharpoons 2\ \text{UDP} \tag{25.}$$

$$\text{GMP} + \text{ATP} \rightleftharpoons \text{GDP} + \text{ADP} \tag{26.}$$

$$2\ \text{GDP} \rightleftharpoons \text{GTP} + \text{GMP} \tag{27.}$$

Although purification ultimately may resolve the difference between the yeast and liver enzyme preparations, only the yeast enzyme catalyzed transphosphorylations between UTP and UMP, and GTP and GMP, in the absence of adenine nucleotide. The nucleosidemonophosphate kinase of yeast catalyzed phosphate transfer to deoxyadenosine-5′-phosphate but not to deoxyguanosine-5′-phosphate. Joklik (99) has described an inosine mononucleotide kinase in yeast, an activity not present in the Lieberman preparation. Muscle adenylate kinase was shown to be specific for 5′-adenylic acid (79, 80).

An enzyme of widespread distribution and high activity, nucleoside diphosphate kinase, was found by Berg & Joklik (100, 101) to effect transphosphorylations of adenosine and uridine pyrophosphates. In muscle a similar enzyme which catalyzed

$$\text{ATP} + \text{UDP} \rightleftharpoons \text{ADP} + \text{UTP} \tag{28.}$$

$$\text{ATP} + \text{IDP} \rightleftharpoons \text{ADP} + \text{ITP} \tag{29}$$

reactions between adenosine and inosine pyrophosphates was found by Krebs & Hems (102). Strominger demonstrated that the pyruvate phosphokinase mechanism which effects the phosphorylation of ADP to ATP will also phosphorylate GDP and CDP to yield GTP and CTP in a system which apparently does not involve intermediation of adenine nucleotides (103).

The phosphorylation of pyrimidine deoxyribosyl-5′-phosphates to di- and triphosphates in unfractionated liver extracts has been reported by Hecht, Potter, & Herbert (104), and reaction mechanisms similar to those operative in the synthesis of ribonucleotide pyrophosphates are implicated.

From the foregoing survey it is apparent that several well-defined enzymic mechanisms for the generation and rearrangement of nucleotide pyrophosphates are known. To these must be added the polynucleotide phosphorylase reaction discussed below (105).

METABOLISM OF POLYNUCLEOTIDES

The mode of action of pancreatic ribonuclease and deoxyribonuclease has been treated extensively in recent reviews (106, 107). While the specificity of ribonuclease in terms of the diester linkages attacked by the enzyme is satisfactorily established, the comparable problem with DNA remains to be solved. Sinsheimer's work (108, 109) has shown the enrichment of deoxyguanylic and 5-methyl deoxycytidylic acids in the dinucleotide fraction of deoxyribonulease digests of thymus and wheat germ DNA; in this fraction a relative increase occurred in the ratio of pyrimidine to purine nucleotide as compared with the native nucleic acid. The preponderant sequence, purine nucleotide-pyrimidine nucleotide, was established for the dinucleotides with the striking exception of cyticylic acid-guanylic acid, which was found to be five times as abundant as its isomer. Sinsheimer points out the contrasting position of 5-methylcytidylic acid by the finding of this compound in the dinucleotide fraction, in the sequence guanylic acid-5-methylcytidylic acid. The deoxyribonuclease of spleen, first described by Maver & Greco (110), which differs from crystalline pancreatic deoxyribonuclease in pH optimum and Mg^{++} requirement, has been found by Privat de Garlihe & Laskowski (111) to cleave fewer diester bonds in DNA than pancreatic deoxyribonuclease and to yield high molecular weight polynucleotide fragments which are susceptible to further degradation by pancreatic deoxyribonuclease. On the basis of this work, the isolation of intermediate molecular weight sequences of DNA, and the refinements of synthetic nucleotide chemistry introduced by the Cambridge group (107) a satisfactory formulation of the mode of action of pancreatic deoxyribonuclease might soon be achieved.

The capacity of phosphodiesterases to transfer nucleotide residues from diester linkages to alkyl hydroxyls and to appropriate nucleotide and nucleoside ribosyl hydroxyls was shown by Heppel & Whitfeld (112) and Heppel, Whitfeld & Markham (113). Ribonuclease, which may be regarded as a phosphodiesterase exhibiting specificity for esters of pyrimidine nucleoside-3′-phosphates, was shown to catalyze the formation of cytidine-3′-methylphosphate from cytidine-3′-benzylphosphate and methanol. The ability of ribonuclease to effect polynucleotide synthesis from the cyclic ester, cytidine-2′, 3′-phosphate, demonstrated for the first time by these workers, marks a departure from the long-held view of the exclusively degradative capacity of this enzyme. Reactions of the following type were found (in the polynucleotide sequence the last member is esterified in the 5′-position and the internucleotide link is 3′, 5′):

$$\text{C-2}', 3'\text{ P} \rightarrow \text{C-3}'\text{ P} + \text{C-P-C-2}', 3'\text{ P} + \ldots \qquad 30.$$

$$\text{C-2}', 3'\text{ P} + \text{Cytidine} \rightarrow \text{C-3}'\text{ P} + \text{C-P-Cytidine} + \ldots \qquad 31.$$

$$\text{C-2}', 3'\text{ P} + \text{Adenosine} \rightarrow \text{C-3}'\text{ P} + \text{C-P-Adenosine} + \ldots \qquad 32.$$

Spleen phosphodiesterase, similar to ribonuclease in that it hydrolyzes

esters of nucleoside-3′-phosphate, but different in being able to attack esters of both purine and pyrimidine nucleotide, was shown to catalyze the formation of alkyl esters from either cytidine- or adenosine-3′-benzylphosphates and methanol. Although spleen phosphodiesterase hydrolyzes cyclic-2′, 3′-phosphates of adenosine and cytidine, from these substrates alkyl esters could not be formed by exchange reactions. The enzyme further differs from ribonuclease by hydrolyzing ribopolynucleotides to nucleoside-3′-phosphates without the intermediate production of cyclic nucleotides. This follows from the demonstration that spleen diesterase hydrolyzes nucleoside-2′, 3′-phosphate to yield nucleoside-2′-phosphate, whereas hydrolysis of ribopolynucleotides by this enzyme yields nucleoside-3′-phosphate exclusively (114). Nucleotide transfer reactions leading to polynucleotide synthesis from cyclic nucleotides could not be demonstrated with spleen phosphodiesterase, but reactions of the type:

$$\text{Adenylic-uridylic} + \text{Cytidine} \rightarrow \text{Adenylic-cytidine} + \text{Uridylic} \qquad 33.$$

were found to be catalyzed by the enzyme. The capacity of both ribonuclease and phosphodiesterase for nucleotide diester transfer reactions, as compared with hydrolytic reactions, is surprisingly high. As the authors emphasize (112, 113), the foregoing reactions effect rearrangements of nucleotide diester linkages within certain specificity restrictions. They do not operate to effect nucleotide diester synthesis *de novo*.

Speculation concerning the synthesis *de novo* of polynucleotide diester linkages has centered about possible reactions involved in the transfer of mononucleotides from pyrophosphate linkage to a 3′-ribosyl linkage (75). The discovery of Grunberg-Manago & Ochoa (105) and Grunberg-Manago, Ortiz & Ochoa (115), that the enzymic reaction in *Azotobacter vinelandii* responsible for exchanging radioactive inorganic phosphate with the pyrophosphate group of nucleoside diphosphates also catalyzed the synthesis of high molecular weight polynucleotides, brought remarkable simplicity, elegance, and clarification to the problem. This reaction, catalyzed by a Mg^{++} requiring enzyme, polynucleotide phosphorylase, effects the reversible phosphorylytic fission of the 3′, 5′-phosphodiester linkage of ribopolynucleotides to yield the constituent mononucleoside-5′-diphosphates. The

$$n\text{X-R-P-P} \rightleftharpoons (\text{X-R-P})_n + n\text{P} \qquad 34.$$

reaction is reversible and ADP, IDP, UDP, CDP, and GDP are converted in high yield to the corresponding polynucleotide, reaching equilibrium when about 60 per cent of the nucleoside diphosphate has disappeared. Not only the synthetic polynucleotides participate in this reaction, but also samples of RNA isolated from *A. vinelandii*, yeast, liver, and *E. coli* have been shown to undergo phosphorolysis by the enzyme to yield GDP, CDP, UDP, and ADP. Nucleoside monophosphates and triphosphates are not reactants. Also, it has been found that equimolar mixtures of ADP and UDP when incubated with the enzyme yield a polynucleotide containing both adenylic

and uridylic acids in a molar ratio of 1:1. A more complex polynucleotide was obtained from mixtures containing ADP, GDP, UDP, and CDP, in the molecular proportion of 1:0.5:1:1 respectively, and both polynucleotides were degraded by ribonuclease to yield products consistent with the well established specificity and action of this enzyme. Molecular weight determinations by the light-scatering method have yielded a value of 507,000 for the AMP polymer and 800,000 for the IMP polymer synthesized by polynucleotide phosphorylase from ADP and IDP respectively. Enzymic and chemical degradations have established that the internucleotide linkages of the polynucleotides are 5′, 3′ phosphodiester bonds, as in native ribonucleic acids.

The importance of this work is great, not only for nucleic acid biochemistry, but for cell physiology as well. In demonstrating an enzymic mechanism for the synthesis of polynucleotide diester linkages *de novo*, Grunberg-Manago & Ochoa (105) made available for investigation in defined systems problems hitherto characterized by discouraging complexity. It cannot be assumed at this time that the polynucleotide phosphorylase reactions will be applicable to all instances of ribonucleic acid synthesis. However, the literature contains evidence derived from experiments with isotopically labeled nucleic acid precursors which is consistent with the general operation of this enzymic reaction. Hurlbert & Potter (116), Hurlbert & Reichard (117), and Potter *et al.* (118), in studies on the conversion of radioactive orotic acid to uridine nucleotides and nucleic acid pyrimidines, present evidence based on the changes in specific activity of these compounds which supports the precursor role of 5′-uridine nucleotides in nucleic acid synthesis. The finding that uridine-5′-phosphate supported the growth of a strain of *Lactobacillus bulgaricus* 09 with a specific requirement for orotic acid, whereas uracil, uridine, and 2′- and 3′-uridylic acids were inactive, might also support a role for 5′-nucleotides in nucleic acid synthesis (119). As pointed out by Leibman & Heidelberger (120), experiments designed to assess the role of labeled nucleotides as precursors of nucleic acids in cellular systems are largely obscured by the extensive and variable rates of dephosphorylation of nucleotides. The experiments of Roll *et al.* (59, 121, 122) have yielded results consistent with this interpretation [also see Brown in (1)]. The utilization of the aglycone of a given nucleotide may be dependent upon the operation of specific dephosphorylation mechanisms, as in the case of the utilization of the purine ring of adenosine-3′-phosphate and not of adenosine-2′-phosphate in *E. coli*, described by Balis, Lark & Luzzati (123). The question now is not whether 5′-nucleotides may be precursors of nucleic acids, but how extensively and under what conditions does the polynucleotide phosphorylase mechanism operate?

Although not yet susceptible to investigation in more or less purified or noncellular systems, the biosynthetic relationships of ribonucleic and deoxyribonucleic acids remain an important but somewhat languishing target of inquiry. An outstanding contribution to this problem was the work of Rose

& Schweigert (124) which showed that the riboside of cytosine, labeled uniformly in the pyrimidine and pentose moieties, was transformed *in vivo* to the deoxyriboside of cytosine in DNA by a mechanism not involving splitting of the riboside linkage or disruption of the pentose. This demonstration of an apparently direct transformation of a ribosylnucleoside to a deoxyribosylnucleoside deserves confirmation and extension. Studies on the origin of deoxypentose in *E. coli* by Lanning & Cohen (125), employing glucose-1-C^{14} as the sole carbon source in culture media of aerobically growing cells, indicate from the molar specific activity of the deoxypentose that the enzymic condensation of glyceraldehyde-3-phosphate and acetaldehyde [the deoxyribose phosphate aldolase reaction of Racker (126)] does not appear to be the only mechanism operating in the synthesis of deoxypentose. This conclusion is based on a calculated molar specific activity of deoxyribose derived from glucose-1-C^{14} by the glyceraldehyde-3-phosphate and acetaldehyde condensation in excess of 76 per cent of that of the labeled glucose of the media, whereas an observed value of only 20 to 26 per cent was obtained. Under similar conditions the deoxyribose of bacteriophage isolated from cultures growing aerobically on glucose-1-C^{14} as the sole carbon source had 41 to 59 per cent of the molar specific activity of the glucose of the medium. The authors suggest that the alternate path of deoxyribose synthesis is restricted in the infected cells. Bearing importantly upon the problem of deoxypentose synthesis is the discovery of MacGee & Doudoroff (127) of the nonoxidative conversion of 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate in *Pseudomonas*. Lanning & Cohen (125) point out that the operation of this mechanism in synthesis of bacteriophage deoxypentose, although not capable of assessment from evidence presently available, would require extensive decarboxylation of glucose-1-C^{14}, a situation not in agreement with actual observations.

Finally, mention should be made of the remarkable advances achieved by Gale & Folkes (128 to 131) in the correlation of nucleic acid and protein synthesis in noncellular systems, which for the first time put this important and controversial field on a sound experimental basis. Gale's Harvey Lecture of 1955 reviews his investigation in this area. The biological role of DNA has been considered in an excellent review by Hotchkiss (132) and by several authors in the 1954 Oak Ridge Symposium on Genetic Recombination (133).

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BIOCHEMISTRY OF VIRUSES[1,2]

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In the preceding five years, the biochemical study of viruses has contributed greatly to a break-through of transcending importance in the understanding of biological interactions and of virus reproduction. The previous era of virus research, invoked in 1935 by the crystallization of tobacco mosaic virus (TMV) and the availability of new methods for characterization of proteins, had led to a physico-chemical approach and had fostered the view that viruses were molecular in nature. Indeed, the two previous chapters in this series by Hoagland in 1943 (1) and by Pirie in 1946 (2) dwelt on the isolation and the physical and chemical characterization of numerous plant and animal viruses. A decade ago only bare mention could be made of the bacterial viruses (bacteriophages) and of biochemical studies of virus reproduction. The changed focus has been brought about by the recognition of the need for biochemical study of well-defined systems and by the concurrent development of tracer techniques and of quantitative biological and biophysical methods. Since detailed reviews of developments in specialized fields of research with plant, animal, or bacterial viruses have appeared intermittently elsewhere, this chapter will attempt the more difficult task of critical evaluation of the progress in the biochemistry of all groups of viruses in the past several years.

The last decade has witnessed at least three major changes in outlook on viruses, the new concepts in large part being predicated on experimentation with bacteriophages. To the biologist, the most significant changes have been (*a*) the recognition of the genetic constitution of viruses [Delbrück (3); Luria (4); Hershey (5); Dulbecco (6); Lederberg (7)], (*b*) the rediscovery of lysogeny [Lwoff (8)], (*c*) the discovery of transduction [Zinder & Lederberg (9)], (*d*) the appreciation of the biological activity of deoxyribonucleic acids (DNA) as genetic determinants and as transforming principles [Hotchkiss (10)] and as carriers of viral activity [Kozloff (11); Hershey *et al.* (12)], (*e*) further study of the biological role of ribonucleic acid (RNA) in plant virus development and protein synthesis [Brachet (13); Gale & Folkes (14)], and (*f*) the mounting evidence for functional and morphological differentiation within the virus particle and for a developmental sequence in viral reproduction (*vide infra*). To the biochemist, a new vista has been opened up in the exploration of the biochemical basis of virus reproduction, the origin of viral components, and the fate of the infecting particle. This is largely based

[1] The survey of literature in this review extends through November, 1955.

[2] The following abbreviations are used in this chapter: DNA for deoxyribonucleic acid; RNA for ribonucleic acid; TMV for tobacco mosaic virus.

on isotopic investigation of virus precursors in the phage system and with TMV. Both disciplines have converged in an attack on the problem of the virus host cell interaction especially in the bacterial and animal systems (6). These advances made through the concerted efforts of workers in many fields have effected a shift from the earlier view of a virus as an autocatalytic molecule. They have led to the concept that the infective form differs from the isolable stable mature form and that in the reproduction of virulent strains the infective form merges with the metabolic apparatus of the cell just as in lysogenic (temperate) types it merges with the genetic apparatus. It remains to be determined to what extent reproductive phenomena are shared in common by such divergent agents as the plant virus nucleoproteins, the bacteriophages, and the viruses pathogenic to animals. However, the prospects for quantitative biochemical and biological investigation of animal viruses are for the first time optimistic because of the recent development of methods for growth in tissue culture and for assay by plaque count [Dulbecco (15); Dulbecco & Vogt (16)].

In order to avoid an annotated bibliography of the papers in the decade since Pirie's review (2), this chapter will be restricted to the recent progress in the physico-chemical characterization of viruses and in the biochemical study of virus reproduction. Fortunately, excellent reviews of the peripheral fields have appeared as cited above and also several symposia and books oriented towards general virology. A new "Advances" series and the first American journal devoted exclusively to virology have just been launched. Hence, most references will be to work of the past few years. Plant, animal, and bacterial viruses may be considered separately or together whichever seems appropriate. Detailed reviews have recently been published on insect viruses by Bergold (17), on phages by Putnam (18), on animal viruses by Sharp (19), and on TMV by Schramm (20). A proper biological classification of viruses does not yet exist. Adams (21, 22) has outlined criteria for the phages, Burnet (23) for animal viruses, and Bergold (24) for insect viruses. Although most criteria are biological, both size and susceptibility to inactivation by physical and chemical agents are useful adjuncts, and a group classification by infrared spectroscopy has even been attempted by Benedict (24). Biochemists should be alert for characteristic viral constituents or metabolic changes induced by infection that might be useful in this regard.

ISOLATION, PHYSICAL AND CHEMICAL CHARACTERIZATION

Isolation of viruses.—As in the study of any biochemical reaction, the first step is still the isolation and identification of the product, a point some investigators have tended to ignore. Despite the introduction of many new techniques, the traditional procedure of "differential centrifugation" has won augmented favor because of the commerical development of refrigerated continuous-flow super centrifuges, preparative ultracentrifuges, and high

speed angle centrifuges. Many adaptations of the centrifugal procedures have been published; most are specific for a given virus. Some modifications include the use of density gradients (25a, 26), field-aligning capsules (27), and swinging bucket rotors, devices that also permit an estimation of the sedimentation constant or of particle size distribution. Salt precipitation, though successful in the crystallization of a number of additional plant viruses [see Markham & Smith (28)], is of little aid when applied to bacterial lysates or to complex animal tissues. Precipitation with ethanol or other organic solvents in the cold is sometimes used alone or in combination with other methods. In certain cases, acidification (29) or dialysis (30) effect a rapid separation.

A number of new physical methods have been explored, but as yet without outstanding success or wide adoption. The most promising of these are zone electrophoresis and chromatography. Puck (31, 32) has discovered the role of inorganic ions in the adsorption of phages and of influenza virus to glass and ion exchangers thus suggesting a principle for the concentration and purification of viruses. Until recently, only plant viruses (about eight in number) had been crystallized. However, the crystallization of polio virus has just been accomplished through a combination of centrifugation and precipitation methods by Schwerdt & Shaffer (33).

The isolation of viruses for biochemical study usually assumes a feasible means of large scale growth. Indeed, generalization on the properties of viruses is hazardous because the systems studied have been selected for their ease of growth and isolation. Plant viruses thus far chosen present no problem. Growth of bacteriophages in liquid culture is now being superseded by the confluent lysis technique. Heretofore, animal virus purification had been difficult because of the small initial amount of virus and the presence of tissue constituents. The outstanding development in the animal virus field is the success of tissue culture growth techniques with HeLa and other types of cells [Sanders *et al.* (34); Weller *et al.* (35); Scherer & Syverton (36); Puck & Marcus (37)] and the introduction of the plaque count assay (15, 16). These advances will permit the isolation of pure lines of viruses (38), the study of one-step growth curves (39), and the application of the isotopic tracer technique so successful with the phages.

Ultracentrifugal analysis.—The second step in the biochemical study of viruses is their physical characterization with proof of purity. The classical methods of protein chemistry together with direct visualization by means of electron microscopy have continued to complement each other. The apt criticism of Pirie (2) and others has led to a less sanguine interpretation of the results of ultracentrifugal and electrophoretic analysis of viruses. However, just as biologists often disregard the need for physical criteria of purity, biochemists often fail to invoke biological criteria such a serological homogeneity, specific adsorbability, infectivity, and plaque or assay type. The current ease of access to the analytical ultracentrifuge is not an unmixed blessing. Owing to the brief period of the run, the schlieren system scans

only material within a restricted size range, and both soluble and aggregated material may escape the notice of the uninformed operator. An infrequent example of the more rigorous type of analysis of ultracentrifugal homogeneity is found in the study of phage T6 by Putnam (40). There is also at least occasional need for a more extended proof of the identity of the infective principle and the characteristic particle in the manner of Lauffer (41, 42).

Although the applicability of ultracentrifugal analysis to even the largest spherical viruses has been established by comparison of sizes obtained with electron microscopy (43), occasional aberrations in sedimentation behavior are encountered. One example is the double boundary phenomenon exhibited by T2 and T6 phages as a function of pH and ionic concentration (19, 40, 44). Aggregation or changes in hydration have been invoked to explain this phenomenon, but no convincing interpretation has yet been offered. However, analyses at varied centrifugal fields have established that the bizarre morphology of the bacteriophages does not produce an oriented sedimentation [Putnam (45)]. A warning about the validity of sedimentation constants of impure viruses is found in the study by Harrington & Schachman (46) of the interaction of TMV and bovine serum albumin. The hydration of viruses is still difficult to estimate; applications of ultracentrifugation and other methods to this problem are reviewed by Lauffer & Bendet (47). Reports of the sedimentation constants of viruses are too numerous to mention. Some tabulations have been made for special groups of viruses (18, 19, 20).

Electrophoresis and diffusion.—Free electrophoresis in the Tiselius apparatus has not proved of much aid in the analysis of viruses with the exception of the plant virus nucleoproteins, e.g., Ginoza & Atkinson (48) have compared eight strains of TMV. As a result of intensive light scattering, the schlieren optical methods are unsuited to the larger animal and bacterial viruses. Only a single electrophoretic study of the phages has been reported so far (49) and only a few of animal viruses. Diffusion measurements face the same optical limitations and have been most misleading when made by biological methods. However, the schlieren method has given important results for T2 and T6 and has established that these phages are not motile (44, 50).

Electron microscopy.—The morphological study of virus particles and of "developing forms" by means of the electron microscope has offered many pitfalls to the unwary virologist. A classical example is the erroneous early identification of bacterial filaments as the poliomyelitis virus, which, in fact, is spherical and was not truly identified in the electron microscope until last year [Taylor & McCormick (51); Bachrach & Schwerdt (52); Sabin *et al.* (53)]. A critical discussion of the interpretation of the electron microscopy of viruses is given by Williams (54). Bang (55) suggests biological criteria for the identification of particles seen by microscopy. The misuse of this unexcelled direct method occurs most commonly with viruses derived from animal

tissues having normal particulate components. However, the unique morphology of the bacteriophages together with quantitative electron microscopy by the particle counting technique have unquestionably established the identity of these viruses and have given the first direct evidence that the minimal infectious unit is one active virus particle [Luria, Williams & Backus (56)].

One must remark on the increasing technical magnificence with which the surprisingly varying morphologies of different types of viruses can now be delineated. However, no attempt can be made to describe the many reports of electron microscopy of individual viruses (many of which have escaped further study). There is a recent complete tabular summary for the phages (18) and several reviews on animal viruses (19, 55), insect viruses (17), and plant viruses (28). The critical point method and the freeze-drying procedure are supplanting the metallic shadow and replica techniques because the former preserve the three-dimensional structure. However, satisfactory methods for specific surface staining or the resolution of internal structure are still awaited. Likewise, the particle counting technique, though applicable to the estimation of the molecular weight of plant viruses, is not satisfactory for the determination of virus purity. A statistical analysis of the particle size distribution of several well-studied viruses is much to be desired. The dimensions reported for viruses face continual revision as the technique improves. Revised values have been given for the "T" set of phages by Noda (57) and by Williams & Fraser (58). It is now clear that all the coliphages, and apparently all phages, possess a tail. The photographic evidence that the tail is the organ of attachment [Anderson (59); Hotchin *et al.* (60)], though plausible, would benefit by evidence that host specificity is strictly obeyed. The crystalline regularity of the phage "heads" as demonstrated by Fraser & Williams (60a) is amazing. Upon osmotic shock, "ghost" forms are obtained (61). Fibrillar strands presumed to be the nucleic acid or chromosomal material of individual particles have been photographed (62). In an elegant adaptation of the quantitative method, Levinthal & Fisher (63) have shown electron micrographs of doughnut-shaped particles presumed to be intracellular precursors of the virus. The morphological dissection of the host cell has also been accomplished through high resolution electron micrographs of sections of *Escherichia coli* made by Birch-Andersen, Maaløe & Sjöstrand (63a). Electron micrographs of the host receptor site for phage T5 have been made by Weidel & Kellenberger (64) who have isolated spherical receptor particles that attach to the virus and inactivate it but only if obtained from a sensitive host strain.

A number of remarkable electron microscope studies of changes within the cell during the development of coliphage have been published recently by Gorrill & Challice (65), Hartman *et al.* (66), Hercik (67), and Levinthal & Fisher (63). Bergold (17) and Morgan *et al.* (68) have illustrated the possible cycle of insect virus growth with the aid of electron microscopy. Comparable studies of plant virus development are not available. However,

Rochow *et al.* (69) have introduced the method of parallel assay by the local lesion and the particle count method. Similar studies of animal virus growth are more difficult to interpret because of the usual presence of inflammation and secondary changes [Bang (55)]. In a representative example, Gaylord & Melnick (70) have exhibited pictures of a tentative time sequence of development for vaccinia, electromelia, and molluscum contagiosum. According to the type of virus studied, either intranuclear or cytoplasmic localization of virus particles has been demonstrated (55). However, there is as yet no fully convincing morphological study of how viruses of any kind reproduce intracellularly (54). All studies are open to the criticism that they may represent selected photographs without evidence of the true time sequence.

Increasing emphasis has been placed on the identification of developing forms of viruses and on pleomorphic forms. However, in no one field is there yet sufficient evidence to illustrate clearly the emerging concepts. The existence of both spheroidal and sperm-like shapes for the virus of avian leucosis and that of Newcastle disease is now attributed by Sharp *et al.* (71) to an artifact of dehydration, the spheroidal particles are regarded as the natural form of the virus. However, filamentous forms are found after growth of influenza or Newcastle disease viruses on tissue cultures of chorio-allantoic membranes. Although there is evidence relating the filaments to the infectious particles, agreement has not yet been reached as to whether the filaments are immature forms of the virus (54, 55, 72). A number of infective and noninfective forms of TMV, all of rod-like nature, have been photographed as well as sub-units obtained by degradation of this virus [Commoner & Yamada (73); Takahashi (74); Delwiche *et al.* (75)]. Jeener *et al.* (76) have discovered two crystallizable, noninfectious forms of TMV that are immunologically related to the virus but lack RNA. These are probably identical with components B_3 and B_6 of Commoner & Yamada (73). The infective and noninfective forms of turnip yellow mosaic virus described by Markham & Smith (28) warrant study at higher resolution. Reputedly immature forms of the phages also have been photographed by Levinthal & Fisher (62) and DeMars *et al.* (77).

Although the evidence for morphologically different forms of intracellularly developing virus needs to be placed on a firmer footing, internal morphological differentiation has been established as a probable characteristic of the mature liberated particles of all viruses. The differentiation may be expressed in various ways, for example, by the presence of membranes as in the T-even phages, insect viruses, and some of the animal viruses. It may appear through unusual shapes as in the spermlike phages or through functional differentiation as in the reproduction of the phages in which the protein membrane is excluded and only the nucleic acid penetrates the host cell (*vide infra*). The concept that viruses are nucleoprotein molecules was suggested by the simple composition and crystallizability of the plant viruses. This view has been dealt a lethal blow by the recent electron micrograph evidence of Fraenkel-Conrat & Williams (78) that the nucleic acid of tobacco

mosaic virus is contained within a cylindrical shell of protein composed of helically arranged subunits.

In summary of a decade of development, one should note the increasing reliance on electron microscopy for the identification of viruses, for evidence of morphological differentiation, and for application to the study of intracellular development. Although only the bacterial phages have a shape unique enough for taxonomy, many viruses have distinctive particles of great uniformity, and a number can be degraded to give evidence of structural and functional localization. Mere description fails to convey the elegant details shown in many of the electron micrographs. However, before being seduced by their apparent clarity, the reader should consult monographs of electron microscopy to discover the artifacts and imperfections inherent in this technique.

Crystallographic analysis.—Detailed x-ray investigation of virus structure is feasible only for the crystallizable plant viruses. The method has yielded some descriptive information on the size and hydration of the spherical plant viruses, but its most important contribution has been to the hypothesis of helical structure. Leonard *et al.* (79) have employed small angle x-ray scattering in a study of the size, shape, and hydration of southern bean mosaic, tobacco necrosis, and tomato bushy stunt viruses. They concluded that the particles are internally hydrated and suggested that a distortion occurs upon the drying incurred in electron microscopy thus leading to a flattening in electron micrographs. Applying the same method to turnip yellow mosaic virus and to its noninfective protein form, Schmidt *et al.* (80) obtained data supporting Markham's view that the protein is identical in size with the virus and has the structure of a hollow sphere within which the nucleic acid is contained (28).

On the basis of x-ray diffraction evidence, Watson (81) has proposed a helical structure for TMV. He suggests that TMV contains a large number of equivalent sub-units with a molecular weight of about 35,000 all being helically arranged around the longitudinal axis. There is further evidence that the RNA forms a central core in TMV (82, 83). Franklin's (84) x-ray data also support the idea of a helical structure of the TMV protein with a helical arrangement of sub-units. A resemblance to this characteristic structure has also been found by Franklin (85) between the repolymerized A-protein and tobacco mosaic virus. Although detailed x-ray studies have not yet been extended to other viruses, Marshak (86) believes that he can discern helical structures in electron micrographs of phages and of polio virus.

Inactivation and disruption.—Investigation of the inactivation of viruses by ultraviolet and ionizing radiation has yielded three kinds of information: (*a*) The most important has been the discovery in the phages of the phenomena of "multiplicity reactivation" by Luria (87) and of photoreactivation by Dulbecco (88), events that have contributed profoundly to the conception of the genetic constitution of viruses. However, the biochemistry of

these phenomena is not well understood; the first appears to be attributable to a genetic recombination, the second to a photochemical change. (*b*) The ultraviolet inactivation of intracellular virus is the basis of one method of studying virus development, the principle of which was subsequently extended by Buzzell *et al.* (89) to thermal inactivation. (*c*) Radiation studies based on target theory have been used by Pollard (90) to deduce the sensitive volume of viruses and to provide indirect evidence for structural and functional differentiation. These methods are largely genetic, cytological, and biophysical. Because the biochemical basis of radiation inactivation is not yet known, original sources and reviews should be consulted (90, 91). This mode of attack has recently been concentrated on the phages. There are reports of protection against ionizing radiation by Bachofer & Pottinger (92), x-ray studies of inactivation by Buzzell & Lauffer (93) and Epstein & Englander (94), cyclotron bombardment by Fluke & Pollard (95) and Pollard & Setlow (96), and of irradiation with low voltage electrons by Davis (97). Action spectra have been deduced for the ultraviolet induction of lysogenic phages by Franklin (98), for latent period extension by Setlow *et al.* (99), for ultraviolet inactivation by Zelle & Hollaender (100), and for photodynamic inactivation by Welsh & Adams (101). In an interesting x-ray analysis Latarjet & Fredericq (102) have identified a colicine with the brushlike appendage of the tail of T6. Pollard's approach of using ionizing radiation methods to ascertain biological structure has been extended to the viruses causing influenza by Buzzell *et al.* (103), to polio by Pollard & Kraft (104), and to Newcastle disease by Woese & Pollard (105). Reports have also appeared on the scattering of ultraviolet light by TMV [Hopkins & Sinsheimer (106)] and of inactivation of TMV by the same source [Klezckowski (107)]. With ultraviolet light, at least, the major effect is on the nucleic acids, and Epstein (108) has claimed a much better identification of the radiosensitive volume with the volume of the nucleic acid rather than with that of the whole virus. The importance of cations in the stability of the phages has been emphasized by Lark & Adams (109), and the reversible inhibition of phages in different media has been carefully analyzed by Ruegamer (110) and by Sagik (111).

A profound influence on the conception of the nature of viruses has been exerted by developments which permit a morphological and functional differentiation of virus activities. Invaluable contributions have been made by the biological study of the successive acquisition of functions by virus particles developing intracellularly, as reviewed by Schlesinger (72) and by Henle (111a). The biochemical approach has been pursued by disruption of the extracellular virus particles with an attempt to ascertain the biological activity of the separate morphological elements and to reactivate the virus by *in vitro* recombination procedures. As yet, there is not good evidence for any correlation of the reproductive forms already described with those artificially produced. Anderson (59, 61), who discovered the osmotic shock properties of the tailed bacteriophages, has concluded that their functional

anatomy is as follows: The DNA is contained within a head membrane that is the first recognized organelle and has the first phage antigen to appear in the infected cell [Lanni & Lanni (112)]. The tail, which is believed to be hollow, terminates in a segment that probably contains the lytic activity (102). When T-even phages in concentrated sucrose or salt solution are suddenly diluted in water, the DNA is liberated leaving behind a protein membrane or "ghost" which retains not only the morphological and serological characteristics of the original particle but also the ability to adsorb specifically to bacteria. The ghosts are lethal for the bacteria but cannot reproduce. Bonifas & Kellenberger (113) have shown that such phage membrane preparations contain a proportion of "killer" particles in which one particle can kill one bacterium. As described subsequently, the reproductive, function seems wholly attributable to the liberated DNA.

Repeated studies of the degradation of TMV have also led to the isolation of the protein and nucleic acid moieties, but the protein is composed of inactive cylindrical fragments. The protein repolymerizes on lowering the pH yielding rods closely resembling TMV (83, 85). The exciting announcement has just been made by Fraenkel-Conrat & Williams (78) that active TMV may be reconstituted from its inactive protein and nucleic acid components. The virus protein is separated from the RNA either by dialysis at pH 10 or by treatment with dodecyl sulfate. Neither the protein nor the nucleic acid fraction is infectious for the tobacco plant, and neither fraction contains particles that resemble the virus in electron micrographs. However, after a mixture of the two fractions is kept at pH 6 in the cold for 24 hr., the solution contains large numbers of particles that are indistinguishable from TMV in the electron microscope and that are infectious for the tobacco plant. Although intracellular reactivation of the coliphages had previously been discovered, this appears to be the first instance of the *in vitro* reactivation of a virus particle from its constituent protein and nucleic acid moieties. The profound significance of this achievement will be better appreciated if reactivation proves successful for other viruses.

Chemical Composition

Nucleic acids.—After isolation and rigid physical characterization, but not before, viruses ought to be submitted to total and to constituent analysis. Whatever separation of functions may in the future be achievable, all viruses so far studied contain both protein and nucleic acid. The latter is either RNA or DNA but rarely if ever both types [see the review by Knight (114)]. Proof of the absence of small amounts of DNA in the presence of RNA (and vice versa) is difficult to establish. If DNA alone has genetic function, this problem bears reinvestigation in the case of the plant viruses because of recurrent reports of the finding of a small amount of DNA in TMV [Holden & Pirie (115)]. The proportion of nucleic acid varies from about 1 per cent in Newcastle disease virus up to 50 per cent in the coliphages but unfortunately has been precisely determined in only a few cases. The discrepancy in even

the elementary analysis of the phages (see 18) is also a reminder of the vexing problem that the chemical composition of viruses has always posed. Since the advent of chromatographic methods, this results more from the failure to remove impurities than from the small amount of material available. In the last decade, little that is new has been learned about the mode of reproduction of viruses from a study of their chemical composition. However, a quite unsuspected qualitative difference has been found in the case of the T-even phages. This is the discovery in coliphages of the hitherto unknown pyrimidine base, 5-hydroxymethylcytosine. This base occurs as a component of the DNA of T2, T4, and T6 phages but is absent from the host and from other T phages [Wyatt & Cohen (116); Wyatt (117)], from Lambda and A_1 phages (8) and from all other nucleic acids thus far examined. Hence, this appears to be the first demonstration of a virus which contains a chemical unit qualitatively different from that of the host. Deoxyriboside and deoxyribotide derivatives from the same source have also been described by Weed & Courtenay (118). Even more startling is the finding by Volkin (119) and Sinsheimer (120) that glucose is present, possibly in a molar ratio of unity with 5-hydroxymethylcytosine. The glucose does not substitute for deoxyribose and is probably attached at the 5-hydroxymethyl position. Further investigation of the stoichiometry of the glucose and of its possible biological significance should be undertaken. Possibly it may play a role in establishing specific strain differences. Cytosine is completely absent from the T-even viruses being replaced by 5-hydroxymethylcytosine (116, 121, 122). The other new pyrimidine, 5-methylcytosine, has not yet been found in any virus (117).

The nucleic acid structure has some latitude. Purine and pyrimidine analogues have been incorporated biologically into viruses although usually with a lethal effect, for example 8-azaguanine [Matthews (123, 124)] and 2-thiouracil into TMV [Jeener & Rosseels (125, 126); Commoner & Mercer (127)] and 5-iodouracil and 5-bromouracil into T2r and T5 phages [Dunn & Smith (128); Zamenhof & Griboff (129)]. Advances in chromatography have permitted analysis of the nucleotide composition of viruses in small amounts. Knight (114) has tabulated data on the molar proportions of purines and pyrimidines for 16 plant, bacterial, and insect viruses as obtained by de Fremery & Knight (130), Dorner & Knight (131), and Wyatt (132), but there is a dearth of such data for viruses which attack vertebrates. The nucleotide composition of viral DNAs is characteristic of the species in that it is demonstrably different for unrelated viruses. However, no significant difference has been found in the comparative nucleotide analyses of strains of viruses in the three cases where data are available, e.g., TMV (130, 133), phages T2, T4, and T6 (116), and insect viruses (132). In general, the virus DNAs reflect the equivalence of adenine to thymine and of guanine to cytosine required by the Watson-Crick (134) helical structure. It will be interesting to learn if the 5-hydroxymethylcytosine and glucose content of

T-even phages is in keeping with the helix deduced by Wilkins *et al.* (135) from x-ray evidence on phage DNA.

Although there are many studies of the physico-chemical properties of viral nucleic acids [see for example the review by Markham (136)], a valuable new approach has been offered by Markham & Smith (28) via structural studies of enzymatically obtained degradation products. Six different end groups were detected in the RNA of turnip yellow mosaic virus, and the maximum mean chain length was estimated to be only 53 residues.

Amino acid composition.—Despite the precise chromatographic methods of recent date, there is a paucity of amino acid analyses except for the plant viruses [de Fremery & Knight (130); Black & Knight (137)]. No unique amino acid analogous to 5-hydroxymethylcystosine has been reported for any virus, nor is there any unusual feature of the amino acid distribution. Comparison of the proteins of different virus strains has been done only within the TMV group of morphologically indistinguishable viruses and for two strains of influenza virus. Unfortunately, the data were obtained by the microbiological method; they have an inherent error of about 5 per cent and in the case of TMV show a drift with the years. Knight (114) believes that definite differences in amino acid composition exceeding 10 per cent accompany virus mutation in TMV and that qualitative changes show up in some less closely related strains. In this type of work, the need for a careful biological control of virus strains is obvious. Extension of this approach to the T-even phages and to animal viruses is desirable.

End-group analysis.—In view of the structural differences now being found in biologically active proteins, investigators should look for more subtle differences than in gross amino acid or nucleotide composition. In truth, they might do well to search for differences in the various protein fractions obtainable from viruses such as the phages. Structural studies of virus proteins are yet in their infancy, and even terminal group analysis has been restricted to the plant viruses. There is substantial agreement that carboxypeptidase exclusively removes C-terminal threonine from TMV in an amount equivalent to one residue per 17,000 gm. [Knight (138); Harris & Knight (139)]. Adding to the evidence for protein sub-units in this virus, is the cysteine content of one residue per 18,000 gm. in all 13 strains (137), the x-ray data (81), and physico-chemical studies of degradation products (139). A dispute exists about the presence of N-terminal residues, Schramm & Braunitzer (140) and Braunitzer (141) claim there is one mole of N-terminal proline per 17,000 gm. whereas Fraenkel-Conrat & Singer (142) discount this finding as spurious owing to liberation of peptide bonds by the end-group method used.

Dethreonized TMV is biologically active (139) as is TMV having 1,000 residues of leucine coupled on [Fraenkel-Conrat (143)]. This fits with earlier data on the reactive groups of TMV as determined by chemical modification [for summary see Putnam (144)]. Knight (138) reports that carboxypep-

tidase liberates different amino acids in smaller amounts from other plant viruses suggesting the presence of several protein chains. The failure of carboxypeptidase to release amino acids from TMV beyond threonine is explained by the presence of the insensitive prolyl bond in the C-terminal sequence PRO-ALA-THR [Niu & Fraenkel-Conrat (145)].

Other constituents.—Constituents other than protein and nucleic acid have often been reported in viruses. Of recent date there is some evidence for acid soluble P and N in the phages (18). The importance of a reinvestigation for minor constituents of possible biological significance has been emphasized by Cooper & Loring (133) in the case of TMV. In many early instances, the claims of miscellaneous constituents may properly be ascribed to impurities, for example, the reported presence of nonpentose sugars in animal viruses. However, the finding of glucose and of 5-hydroxymethylcytosine in the T-even phages stands as a warning, for prior reports of nonpentose sugar had been abruptly dismissed. Lipides are thought to be present in all animal viruses but to be absent in plant viruses. It would be desirable to investigate their occurrence and function more fully.

A return to the precise and systematic analysis of viruses is needed with figures based on total N content. As yet, the amino acid composition of only one of the T3 phages by Fraser & Jerrel (146) has been given despite the need for comparative studies. Terminal group analysis offers an invaluable new approach to the structure of viruses. A more discriminating application of chromatographic methods of purine, pyrimidine, and amino acid analysis may possibly reveal other unsuspected and unique constituents such as 5-hydroxymethylcytosine.

REPRODUCTION OF VIRUSES

The past few years have witnessed more important developments in the biochemical study of the reproduction of viruses than in their physical and chemical characterization. It is now considered that the isolated virus is in a "resting phase" without detectable metabolic activity, whereas biological concepts, such as "life cycle," "developing forms," "mating," "vegetative stage," and "germinal substance," have been applied to the reproductive process. Luria (4) has given an operational definition of a living substance or organism within which the viruses fall. Bergold (147) states, "The majority of virus workers of today believe, even whether they say it or not, that viruses are organisms." If so, they are organisms devoid of demonstrable enzymatic function in the extracellular particle for with two possible exceptions no claim of enzymatic activity in a highly purified virus has been generally acknowledged. The most widely documented case is influenza virus, which has a mucinase or "receptor destroying property" [Burnet (148); Lanni & Lanni (149)], the significance of which is still questioned by some authors [Bauer (150)]. The second case is avian erythromyeloblastic leukosis virus, which dephosphorylates both adenosine and inosine triphosphates [Green & Sharp (151); Green (152)]. Thermal studies of the rate of inactiva-

tion of the avian virus have yielded evidence for the lack of identity of the virus particle centers responsible respectively for infectivity and enzyme activity in preparations obtained by a single ultracentrifugation cycle [Eckert *et al.* (153)]. Bauer (150) has discounted earlier accounts of enzymatic activity in vaccinia and has concluded that animal viruses possess no enzymatic activity apart from the instance of mucinase in influenza virus. Enzymatic activity has not yet been seriously ascribed to the plant viruses. However, Lark & Adams (109) have suggested that the tip of the tail of T5 phage is composed of a calcium activated enzyme implicated in the injection of the nucleic acid. Christensen & Tolmach (154) believe that the irreversible phase of the attachment of T1 to the cell wall is an enzymatic event, but do not impute the activity to the virus. It is interesting that Latarjet & Fredericq (102) have by an indirect method located a colicine (bactericidal activity) in the tip of the tail of T6.

Adsorption and invasion.—Until recently adsorption, the first phase in the growth cycle, had been studied as a kinetic process with an inexplicable specificity ascribed to a template factor. The first significant advances in the understanding of the biochemical basis of adsorption and invasion have been: (*a*) the recent discovery, already adumbrated, that the process of specific adsorption of phages may be studied in an isolated system containing incomplete virus particles ("ghosts") or incomplete host cells (membranes and "receptor spots") and (*b*) the evidence derived by Puck and collaborators (31, 32, 154) that the attachment of viruses to specific host cells involves the institution of electrostatic bonds between sites on the surfaces of the two bodies. The interaction of intact phage with cell membranes leads to the disintegration of the cell walls with the liberation of nonsedimentable N both from the membranes and the virus [Barrington & Kozloff (155)], and the interaction of phage ghosts with intact bacteria leads to a cessation of growth and lysis of the cell [Barlow & Herriott (156)]. The sequence of steps in viral invasion and the separation of the functions of adsorption, virus splitting, and cell killing have been most successfully explored by Puck and co-workers (157, 158, 159) and by Garen (160). They have concluded that the primary attachment consists of the "formation of reversible electrostatic bonds between sites on the virus and a portion of a repeating set of charge groups, which forms a gene controlled pattern covering the host cell surface" (31). The effect of inorganic cations in adsorption is greater on the virus than on the cell. This conclusion is based on: (*a*) the salt requirements for phage adsorption (32, 110) and (*b*) the ability of the T phages and of influenza virus (but not of *E. coli*) to become attached to cationic exchange resins with the same specific salt requirements as for cell attachment (32). The effects of surface substitution with chemical group reagents suggest that the positively charged amino groups of T2 virus bond with complementarily situated carboxyls on the cell surface but that both types of charged groups may be involved from each surface in the case of T1 (159).

Although the primary electrostatic interaction of *E. coli* with T1 and T2

is diffusion controlled and is completely reversible, it is followed by a second nonenzymatic step consisting of irreversible virus breakdown. Cell killing also appears to be nonenzymatic. According to Puck's picture (157, 158), penetration of viral DNA occurs through action of a cellular enzyme forming a local hole. This is followed by a sealing phenomenon that results in "mutual exclusion" (the inability of unrelated viruses to penetrate and multiply) and "superinfection" (the breakdown of secondarily added viruses of the same type). The failure of the sealing mechanism to keep pace with the lytic reaction results in "lysis from without" (lysis of cells at high virus to cell ratios). Unlike the sealing reaction, neither adsorption nor the lytic reaction appears to require operation of the energy-yielding mechanism of the cell, for they occur with cell membranes as well as with heat-killed or ultraviolet inactivated cells. The extension of these studies to other viruses is greatly to be desired. A beginning has been made in the observations on the attachment of influenza virus to cationic resins (32).

Metabolic Activity of the Infected Cell

Many investigators have unsuccessfully sought for some change in the enzymatic activity of the infected cell that might give a clue toward explaining the over-riding influence of the virus on cell metabolism. Most attention has been focused on nucleic acid and protein synthesis and on amino acid, phosphorus, and nucleotide metabolism. Workers have sought to find both chemical and enzymatic changes in the infected cell. They have looked for the acquisition of new functions and for the synthesis of new metabolites. However, thus far, metabolic alterations wholly ascribable to virus infection have been found only in the *E. coli* bacteriophage systems. Such changes, if they occur in animal systems, are probably obscured by secondary processes, such as inflammation, and they are difficult to observe in plant tissue. Bauer (150) has summarized a variety of studies of the changes produced in tissues following virus infection. There is a clear need for model systems for biochemical study; such opportunity in now afforded by the tissue culture methods of animal virus growth. Heretofore, intact cells have been considered essential for virus multiplication. However, Salton & McQuillen (161) and Brenner & Stent (162) have just demonstrated independently that phage growth can occur in protoplasts of *Bacillus megaterium*. The isolated cell walls specifically adsorb the phage whereas the protoplasts do not. Hence, it is necessary first to infect the intact bacteria and then to convert the infected cells into protoplasts by lysozyme treatment. It appears that the protoplasts are also capable of reproduction by division [McQuillen (163)]. These findings represent an important advance towards the goal of biochemical study of viral growth in sub-cellular structures.

Enzymatic changes.—The reputed inability of phage-infected bacteria to form enzymes adaptively has been taken as evidence that virus development inhibits the synthesis of new enzymes and normal bacterial protein. Benzer (164) has recorded yet another case wherein infected *E. coli* failed to develop

enzymes adaptively [in contrast see Joklik (165)]. Several instances of the acquisition of new metabolic capabilities upon phage infection have been reported for lysogenic systems, for example, transduction (9) and the conversion of nontoxigenic *Corynebacterium diphtheriae* to toxin production [Groman (166)]. However, there is only one known instance of the induction of a new metabolic capability in systems wherein infection leads to the inhibition of cell multiplication and where virus synthesis becomes the dominant metabolic activity; namely, the induction of an active net synthesis of thymine and of 5-hydroxymethylcytosine upon T2 infection of a thymine requiring mutant of *E. coli* [Barner & Cohen (167)]. Neither pyrimidine is synthesized appreciably in uninfected, thymineless organisms. The mode of synthesis of the virus specific DNA which contains 5-hydroxymethylcytosine is of great interest. Rosenbaum *et al.* (168) have found that although chloramphenicol stops both induced enzyme biosynthesis and net protein synthesis in *E. coli*, viral characteristic DNA appears in chloramphenicol inhibited cells infected with T2. This suggests that either the virus carries the necessary enzymes to make 5-hydroxymethylcytosine or that they are already in the cell.

Changes in enzymatic activity upon infection appear to be nonspecific and indirect except with regard to nucleic acid metabolism. The situation in the coliphage system is not clear. One might anticipate both qualitative and quantitative alterations in the activity of enzymes involved in nucleic acid metabolism, for host DNA is degraded for transfer to virus progeny (18, 169), and DNA containing a new kind of pyrimidine is being formed. Friedman & Gots (169a) have, in fact, reported a marked decrease in enzymatic degradative activity towards substrates involved in nucleic acid metabolism. However, coupled with this, there is a general lag in degradative enzymatic activities, e.g., deamination, phosphorolytic reactions, etc. Since a multiplying and a nonmultiplying system are being compared, it is difficult to ascertain how much difference is attributable to growth. Joklik (165) observed that certain oxidative enzymes were unaffected whereas others were inhibited and only formic hydrogen lyase was stimulated. Pardee and coworkers (170) investigating the activity of nine bacterial enzymes in infected *E. coli* reported that only deoxyribonuclease activity was enhanced. Kozloff (11), who independently discovered the increase in deoxyribonuclease activity of phage infected cells, has traced this to destruction of a specific deoxyribonuclease inhibitor. The inhibitor was identified as a particular kind of RNA in the cell. Thus, the major quantitative change in enzymatic activity upon phage infection is ascribable to release of an inhibitor, and the enzymatic pathway for the qualitative change (5-hydroxymethylcytosine synthesis) remains to be elucidated.

The search for altered enzymatic activities upon infection with animal viruses has been even less successful. Pearson (171) has summarized some of the earlier studies. Smith & Kun (172) have made a particularly careful study of the changes in the chick chorioallantoic membrane following infec-

tion with a variety of animal viruses. In all cases, there was an increase in anaerobic hexose diphosphate breakdown. This did not occur as a result of inflammation but as a part of the increased glycolysis.

Chemical changes.—Chemical changes following infection with animal viruses usually are obscured by inflammation and cellular degradation. Work has been centered on amino acid and P distribution, but net changes are seldom found. Hence, resort must be made to use of the isotopic method (*vide infra*). For example, Johnson *et al.* (173) observed no important changes in amino acid levels in chorioallantoic membranes infected with mumps or influenza virus. However, TMV synthesis in isolated leaf disks does induce transitory changes in the free amino acid balance that Commoner & Nehari (174) believe are associated with the early synthesis of a nonvirus protein. Marked changes also occur in the electrophoretic pattern of the cytoplasmic proteins of plants infected with certain viruses [Wildman, Cheo & Bonner (175)].

A profound alteration in P, N, and carbohydrate metabolism has been demonstrated in the *E. coli* bacteriophage system by Cohen and others (175a, 176, 177). Cohen made the striking observation that in cells infected with the T-even phages RNA production ceases abruptly whereas DNA synthesis is stimulated about threefold. After isotopic studies by Kozloff (11), Putnam (18) and Evans (169) demonstrated the conversion of bacterial DNA to viral DNA. Hershey, Dixon & Chase (121) exploited the discovery of 5-hydroxymethylcytosine as a means of permitting a distinction between the synthesis of the two kinds of DNA. They confirmed that the characteristic bacterial DNA is decomposed after infection whereas the cytosineless viral DNA increased progressively in two forms, one consisting of infective particles and one not. The synthesis of phage-related materials has also been followed during infections aborted by proflavine [DeMars (178)]. The absolute cessation of RNA synthesis upon infection with the virulent coliphages is in contrast to the continued residual growth and synthesis of RNA after induction of lysogenic *B. megaterium* [Siminovitch & Rapkine (179)].

The most significant biochemical finding in coliphage reproduction is the shift in P metabolism manifested by the total inhibition of RNA synthesis and the acceleration of DNA synthesis, the phenomenon designated as the phosphorus shunt. Cohen & Roth (175a) conclude that although glucose utilization via the oxidative pathway is sharply curtailed in virus infected cells, inhibition of the phosphogluconate pathway is not the immediate cause of the total inhibition of RNA synthesis. Lanning & Cohen (177) have shown that there must be two mechanisms of deoxyribose synthesis in *E. coli*, for on T2 infection, the deoxyribose synthesized from glucose 1-C^{14} has twice the specific activity of the same pentose in a normal cell.

Nutritional studies of virus growth are difficult to perform to the exclusion of an indirect effect on the host cell. Much of this work involves the use of antimetabolites and has been reviewed both for the phage system (18) and for animal viruses (171). Suffice it to say that although additions of purine,

pyrimidines, amino acids, or vitamins may sometimes influence viral growth no specific factor has yet been found which is intimately and solely required for reproduction. With the coliphages, dissociation in the synthesis of DNA and of protein can be produced by methods leading to abortive virus production (77, 178). Through use of amino acid requiring mutants of *E. coli*, Burton (180) has endeavored to study the separate aspects of protein and nucleic acid synthesis and has shown that the synthesis of phage DNA is dependent on the initial synthesis of proteins. One salient observation stands out; namely, contrary to the hypotheses about the controlling role of RNA in protein synthesis, phage protein is formed despite the cessation of RNA synthesis and turnover.

Energy requirements.—Since there is no creditable evidence for the existence of respiration or any other energy producing mechanisms within viruses, it is assumed that the energy needed for virus synthesis is generated by the infected cell itself. Thus far, all results of the study of energy requirements for synthesis of viruses have been interpreted in terms of well-known metabolic pathways. Through use of malonate, fluoroacetate, and 2,4-dinitrophenol, Ackermann & Johnson (181) and Ainslee (182) have implicated the Krebs cycle as a possible source of the energy for virus multiplication. From work with similar inhibitors, Moulder *et al.* (183, 184) have concluded that the energy for the synthesis of pneumonitis virus is probably supplied by high energy phosphate bonds generated by the aerobic oxidation of endogenous substrates. The energy for phage synthesis also appears to be a product of host enzyme activity shunted from use in bacterial synthesis (175a, 176, 177). It is clear that virus infection leads to a redirection of host metabolism although the obligate contribution of the host may vary from virus to virus. The formation of 5-hydroxymethylcytosine represents the only instance of the synthesis of a new metabolite, and the enzymatic mechanism for this remains to be elucidated.

Many other interrelated facets of intracellular virus development have been cut in the past decade. Beyond our scope are the following valuable approaches: (*a*) immunological study of developing antigens [e.g., Lanni & Lanni (112)]; (*b*) cytological observations on phage development [e.g., Hartman *et al.* (185, 186)] and viral development in isolated animal tissues [e.g., Ackermann & Francis (187)]; (*c*) one-step growth curves, this method so successful in the study of the phages can now be extended to animal viruses susceptible to assay by the plaque method [Dulbecco (38); Cooper (188)]; (*d*) optical measurements on infected cells; (*e*) radiobiological analysis; and (*f*) studies on abortive infection with phages and synchronization of the cell growth cycle [reviewed by Adams (189, 190)]. Schlesinger (72) and Henle (111a) have reviewed the more biological approach to the study of developmental forms of viruses. The more philosophical and ecological approach involving the elusive relationships among virus, pro-virus and the genetic apparatus of the cell have been dealt with by Luria (4), by Lwoff (8), and by Lederberg (7). Although invaluable information regarding the

mode of virus action has been obtained by the use of metabolic inhibitors, this subject has already been thoroughly reviewed by Pearson (171) for the propagation of plant and animal viruses and by Putnam (18) for the phages, and Matthews & Smith (191) have discussed the chemotherapy of viruses.

Isotopic Studies

Even in retrospect, the swift advance in biochemical comprehension attributable to isotopic studies of virus development is surprising. Several once current hypotheses have had to be abandoned. For example, both in the case of the coliphages and TMV, the isotope data have clearly shown that no macromolecular precursor exists within the cell that is catalytically converted by infection to yield new virus. Rather, there is a dynamic state of metabolism in which virus particles are continuously formed from pools of low molecular weight intermediates derived from external sources by normal synthesis and by degradation of host material. Also, the idea that the whole virus particle is a template must be discarded, at least in the case of bacterial viruses, because the isotope studies have unambiguously demonstrated that the virus is broken down and only a portion enters the bacterial cell. This reproductive or germinal form contains little or no protein. Hence, both specificity of form and hereditary structure must largely or wholly be associated with the virus nucleic acid. This leaves a dilemma since phage protein is initially produced in the absence of a proteinaceous template. The tracer methods have permitted the study of two problems heretofore unapproachable, namely, (*a*) the origin of viral constituents and (*b*) the fate of the infecting particle. Although the isotopic studies of phage reproduction have now perhaps reached fruition, similar research on animal viruses has hardly been initiated.

Origin of viral constituents.—The tracer approach to the origin of viral constituents was introduced by Cohen (192) who showed that phosphorylated phage constituents are synthesized in the main from inorganic phosphate assimilated from the medium after infection. The Chicago group, led by Evans, (193) focused attention on the host contribution. In a series of experiments with P^{32} (11, 194, 195, 196), with N^{15} (195, 196, 197), with C^{14} purines (198), and with C^{14} lysine (197), it was shown that about one-third of the P of T6 phage and about one-quarter of the N originates in components of the host cell laid down prior to infection whereas three-fourths of the P and N of the small virus T7 originates in the same source. These observations, confirmed by Labaw (199) for the P of other T viruses, led to the hypothesis that host DNA is converted to virus DNA (18, 193). The initial evidence for the experimental verification of this hypothesis has been reviewed by Evans (169) and by Putnam (18). Support for the hypothesis is found in the results of balance and kinetic experiments in which the cells were doubly labeled with N^{15} and P^{32} (194 to 198). The data indicated that most of the bacterial DNA was transferred to the virus, but it appeared that prior to the

synthesis of the phage DNA, host DNA was degraded to fragments such as deoxyribonucleotides. Together with the work of Koch on acid soluble purine metabolism (198, 200), these studies have established that adenine and guanine are transferred intact from the host to the phage and that the amount of acid soluble purines is insufficient to account for the host contribution. The host contribution of pyrimidines to virus DNA was clarified by the discovery of 5-hydroxymethylcytosine after which Gollub-Banks (see 11) found that a transfer of host thymine takes place without conversion to 5-hydroxymethylcytosine. Subsequently, Cohen & Weed (176) demonstrated that host cytosine is a major host precursor of virus 5-hydroxymethylcytosine. Since preformed RNA is inert in the virus infected cell and host DNA is the only significant source of preformed thymine, it is evident that intact purine and pyrimidine bases are derived from the host DNA. The host DNA must be degraded to nucleotide units prior to incorporation into phage DNA. This follows, from the difference in composition of the two DNAs, from the results of experiments with doubly labeled host cells (194, 195, 197, 198), and from the quantitative similarity in the transfer of P, nucleotide bases, and deoxyribose (177). The degradation of host DNA prior to use for virus DNA is consistent with observations made by Hershey (5, 121) on the kinetics of 5-hydroxymethylcytosine formation and by Burton (180) on the effect of the omission of purines during the early stages of phage development in purine-requiring mutants. Although virtually all the host DNA is used for virus synthesis, the specific activity of the virus DNA is always diluted by net synthesis. Hence, in the case of the large T-even phages, three-quarters of the viral DNA is newly formed from the inorganic compounds and the carbon source of the medium but only one-quarter in the case of the smaller T viruses.

The painstaking attempt to establish the transfer of host DNA to virus DNA might seem belabored but for the fact that nuclear degradation and a quantitative utilization of the host DNA are far-reaching consequences of the phage infection. However, a series of kinetic studies have established that there is no specific obligate transfer of a moiety of host DNA to each virus particle. From analysis of the specific activity of the pyrimidine nucleotides to the virus yield from early lysing cells compared to those in which lysis was delayed, Weed & Cohen (201) deduced that the first virus particles formed contained all the pyrimidines transferred from the host. After thorough study of the kinetics of P^{32} assimilation in T4 phage, Stent & Maaløe (202) decided that the earlier formed virus particles receive a greater proportion of their P from the host than do the later ones. In other words, all phages do not receive an equal measure of P or nitrogenous bases. Stent & Maaløe concluded that there exists a pool of P compounds into which flows P from bacterial constituents and from P assimilated after infection and from which phage P is derived. Hershey and co-workers (5, 12, 121) reached similar conclusions after an ingenious use of 5-hydroxymethylcytosine as an analytical marker of the intracellular viral DNA as well as by resort to P^{32}.

They also made the interesting observation that P in mature phage does not exchange with P in the precursor, showing that maturation is an irreversible process.

In contrast to the dynamic state of DNA metabolism within *E. coli* infected with virulent phage is the quiescent state of RNA. Cohen (192) first observed the lack of turnover of RNA phosphorus, an observation that has been confirmed several times (198, 203, 204). To be sure, Hershey (5, 121) has suggested that a small fraction of the RNA might undergo moderate turnover, and RNA synthesis might thus keep pace with DNA synthesis. However, Manson (203), studying the incorporation of marked glycine into the purines of a glycine-requiring mutant of *E. coli*, has concluded that RNA is "a metabolic dead end." Although Lanning & Cohen (177) pose the question of the conversion of ribose to deoxyribose, there is no evidence for this in *E. coli*. It should be borne in mind that the dynamic state of metabolism established for mammals does not pertain to bacteria. Hershey's conclusion (205) that nucleic acids are not degraded in growing bacteria is strengthened by the experiments of Koch & Levy (206) on the differential labeling of nucleic acid purines. The same workers were unable to detect degradation of protein in growing uninfected bacteria. Hence, the degradation of host DNA in infected cells is to be added to the growing list of qualitative changes induced by the virus. As Hershey *et al.* point out (12):

> Infection alters DNA metabolism in three ways: It stops formation of bacterial DNA instantly; it quickly initiates the synthesis of viral DNA; and it causes the existing bacterial DNA to all but disappear. These facts suggest that the rapid conversion of bacterial DNA into viral DNA is probably quite as unique as the accompanying genetic events certainly are.

The extensive studies on the synthesis of virus DNA have no counterpart in the formation of phage protein. Most of the protein of T6 is synthesized *de novo* from the components of the medium. Experiments with N^{15} (204) and with C^{14} lysine (197) have revealed that only about 10 per cent of the viral protein N of T6 is derived from host protein. The results with lysine indicated that the small host contribution originates in the bacterial protein rather than in the pool of free amino acids. Nor does bacterial protein significantly contribute N for synthesis of viral nucleic acid (198). Unlike the case with T6, almost half the protein N of the small phage T7 is derived from host sources (195).

Although RNA synthesis ceases on infection and the formation of viral DNA undergoes a slight time lag, Cohen (207) finds that protein synthesis goes on unabated in viral infected cells. Unfortunately, no specific marker for the detection of viral protein synthesis exists comparable to 5-hydroxymethylcytosine for DNA. The question arises whether protein synthesis is necessary for the replication of the viral DNA. From study of the incorporation of inorganic S^{35} into the protein of infected cells and its subsequent appearance in mature phage, Hershey *et al.* (12) conclude that the infected

cell forms two classes of protein, one viral precursor and the other not. At a later stage, the synthesis of viral precursor protein predominates. Burton (180), using amino acid requiring mutants, has obtained evidence that proteins must be synthesized which later initiate the synthesis of viral DNA but do not form a part of the phage structure. Clearly, the pathways of protein and nucleic acid metabolism diverge early in phage reproduction and do not again transect until maturation of the complete particle. Further investigation of the sequence and interaction of viral protein and nucleic acid synthesis in phage reproduction is merited.

Tobacco mosaic virus.—Little is known about the origin of viral nucleic acid in the case of TMV, for owing to unfavorable experimental conditions the early investigations were abortive. However, a significant analogy to phage reproduction is found regarding the precursors of TMV protein. Wildman, Cheo & Bonner (175) had proposed that TMV is synthesized from a pre-existing normal protein detectable by electrophoretic analysis and comprising up to 50 per cent of the soluble leaf protein. However, Meneghini & Delwiche (208) found that in tobacco leaves infiltrated with $N^{15}H_4Cl$ and inoculated with virus, the isotope appears much more rapidly in the isolated virus than in normal cytoplasmic protein. This evidence that the virus is not formed from a precursor protein received support in the observations of Commoner *et al.* (174, 209). Further study with N^{15} by Commoner, Scheiber & Dietz (210) indicated that ammonia is the major and perhaps the only component of the pool of free nonprotein N, which participates in *de novo* synthesis of TMV or its precursor. This leads to the dilemma that the amino acid residues of TMV protein are apparently not derived from the corresponding free amino acids in the host cell. It would be desirable to investigate further the manner in which the ammonia is incorporated into the TMV protein. Nevertheless, it is significant that in TMV formation as in phage synthesis, the protein is produced *de novo* from low molecular weight constituents by a diversion of some part of the host protein synthesizing apparatus from its normal course. Jeener (211) proposes that there is also a close analogy between the synthesis of phage and of turnip yellow mosaic virus in that the nucleic acid and protein fractions of the viruses are synthesized independently and later united. Exposure of infected plants to $C^{14}O_2$ led to a greater specific activity in the noninfective protein antigen than in the active virus. This suggests that the antigen may be the protein moiety of the incomplete virus. However, the nonvirus proteins B_3 and B_6 are probably not precursors of TMV protein in the temporal sense (209).

Animal viruses.—Unfortunately, comparable data on viral protein and nucleic acid precursors are not available for animal virus systems. To be sure, isotopes have been used to study the growth of viruses capable of cultivation in the embryonated egg or in mouse brain. The problems of tracing virus synthesis under these conditions are illustrated by a study of the effect of Theiler's GD VII virus on the uptake of P^{32} or C^{14} from minced mouse brain. Moldave *et al.* (212, 213, 214) have observed that the uptake

in vitro of P^{32} into the phospholipides, protein, and RNA fractions of mouse brain is stimulated by the infection. A similar relationship was found between virus growth and the amino acid metabolism of the infected tissue. An attempt to localize the effect in the various subcellular fractions of the brain revealed that the most pronounced stimulation was on P^{32} incorporation into the protein and nucleic acid fractions of the smallest particulate fraction. Similarly, infection of the chick embryo with feline pneumonitis virus causes no demonstrable change in the distribution of P in the yolk sac fractions. It does, however, cause the yolk sac to incorporate P^{32} into its DNA at a greater rate than that of normal tissue [Zahler & Moulder (184)]. This virus appears to contain both RNA and DNA, and both nucleic acids had higher specific activities than any chemical fraction of the yolk sac pool. This is interpreted as a result of the induced synthesis of virus nucleic acid at a time when the acid soluble P of the yolk sac had a very high specific activity. Not all the virus induced nucleic acid is believed to be incorporated into the final virus yield. This hypothesis parallels some of the current ideas on bacteriophage multiplication in that it suggests that the nucleic acids of the virus are laid down early in the infection before the appearance of the mature infectious virus particle.

The fate of the infecting virus particle.—One of the most significant accomplishments of modern virus research has been the elucidation of the fate of the infecting phage particle. Nonrecoverability of the infecting virus inoculum had been reported in the psittacosis, influenza, and phage systems and appeared to be a general phenomenon. The first direct chemical evidence on the fate of the infecting virus particle and on the transfer of constituents from parent to progeny was obtained in 1950 by Putnam & Kozloff (215) who infected *E. coli* with T6 labeled with P^{32}. They found that up to 40 per cent of the parental P appeared in the progeny virus and concluded that the original particle does not survive infection. This raised numerous questions and stimulated many workers to the investigation of two basic problems: (*a*) the significance of the breakdown of the infecting particle during invasion and (*b*) functional differentiation and genetic continuity in relation to the transfer of parental material to subsequent generations.

Breakdown of the infecting particle.—In this study the isotopic marker is incorporated into the virus particle, and its liberation in various forms is measured following adsorption. Kozloff (216), employing T6 labeled with N^{15} and P^{32}, found that every infecting virus particle breaks down releasing unsedimentable N^{15}. He concluded that most of the parental N like much of the P is not contributed to the progeny. However, when a variety of T phages labeled with P^{32} were studied under somewhat different growth conditions, French, Graham *et al.* (217, 218) discovered the phenomenon of "stimulation breakdown." In this concept, the primary infecting particle reorganizes the cell, perhaps by redirection of deoxyribonuclease activity, so that secondary adsorbed particles are broken down to a greater extent and excluded from the

cell. Mackal & Kozloff (219) and Christensen & Tolmach (154) have elaborated on the possible steps in this invasive process.

Nature of the parental contribution to progeny.—Quantitative studies of the parental contribution to progeny indicate that from 10 to 50 per cent of the infecting viral nucleic acid N, C, and P is contributed to progeny, but less of the N and none of the S appears in the progeny protein. This alone indicates an extensive chemical rearrangement and excludes a transfer via intact virus. Maaløe & Watson (220), Hershey (5, 12, 221), and French *et al.* (218) all confirm the original observation (215) of a transfer of about 35 per cent of the parental P, and C^{14} labeled parental adenine is transferred to about the same extent (222). To be sure, the values reported for P transfer vary somewhat and appear to be lower for the small phage T7 (219). Kozloff (223), using doubly-labeled T6, first observed that relatively more parental P than N was contributed to the progeny nucleic acid and that the parental N appearing in the progeny nucleic acid always exceeded that in the progeny protein. Hershey & Chase (224) established a functional differentiation of the viral nucleic acid and protein moieties by showing that viral S^{35} (protein) was virtually excluded from the cell whereas much of the P^{32} (nucleic acid) penetrated. More recently, French (225) has confirmed the virtual exclusion of viral protein using C^{14} lysine labeled virus for infection, and Hershey (226) has set an upper limit of about 3 per cent for the protein content of the "germinal substance" of T2 phage. These results, when added to other obervations already described, indicate that viral protein and nucleic acid have independent functions in multiplication. The protein membrane has an adsorptive capacity with lethal and lytic powers. The nucleic acid presumably has the whole hereditary function, and the function of the antigenically distinct protein of the "germinal substance" is unknown. However, it is not an important precursor of the viral offspring.

Distribution of parental contribution in the progeny.—The question whether the parental nucleic acid contribution has any special function or specific location in or distribution among the progeny particles has been attacked in several ways. It was first thought that transferred DNA might be a conserved unit having hereditary characteristics, the remainder of the parental DNA being considered dispensable. This hypothesis was refuted by the "second generation" type of experiment in which it was shown that the first descendant virus particles likewise transmitted one-third of their P to their progeny (218, 220). This finding indicates that the parental P contribution was located at random, else it would have been transferred as an intact unit.

Since the transmissible P is not located specifically in hereditary units passed on intact from parent to progeny, it is improbable that it would be distributed uniformly among all the progeny which themselves are formed intracellularly, possibly in a logarithmic sequence. The first evidence that each virus particle is not endowed with an equal share of parental substance

was deduced by Kozloff (223) from a study of the N^{15} abundance as a function of virus yield from N^{15} labeled infecting particles. Watson & Maaløe (222) and French *et al.* (218) studied the P^{32} distribution in the virus yield from prematurely lysed cells infected with labeled T2. They concluded that the transmitted P was largely incorporated into the first virus particles formed out of the single generation comprising some several hundred phages. Stent & Jerne (227) subsequently presented evidence that the transferred P atoms of parental DNA are distributed over at least 8 but no more than 25 of the progeny. This ingenious work is based on the finding of Hershey *et al.* (228) that the "efficiency of killing" by radioactive decay of P^{32} incorporated into phage is 0.10, i.e., on the average, at 4°C. (229) 10 internal P^{32} disintegrations kill the virus particle. Here, the interpretation was made that only one disintegration in ten is lethal; in other words, only 10 per cent of the P atoms of each phage particle are essential for hereditary structure and infectivity. Since neither the transferred P atoms nor those whose decay leads to inactivation appear to reside in any "special fraction" of the phage (229), Stent and Jerne were able to exploit the decay of transferred P^{32} atoms to study the distribution of parental P among the progeny.

The genetic significance of the transfer of nucleic acid from parent to progeny continues in dispute. Both Kozloff (223) and Watson & Maaløe (222) have found a transfer of P^{32} from one strain of labeled virus to another, although the pair of viruses cannot multiply within the same cell. This transfer must be nongenetic and probably arises from the availability of phosphorus atoms as a result of stimulation breakdown of the virus particles. Moreover, inactivation of the parent phages by ultraviolet light or x-rays does not greatly alter the contribution of parent material to viral progeny (222, 223). These challenging experiments present the first chemical evidence for transfer of material from parent to progeny, but the nonspecific utilization of the parent nucleic acid N and P implies that form, not substance, is the essential characteristic which the parent particle bestows on the progeny.

The isotopic investigation of the fate of the infecting virus particle in plant and animal systems is greatly to be desired. Although the earliest studies with TMV were abortive, a new beginning has been made by Jeener & van Rysselberge (230) who labeled TMV with P^{32} and C^{14} and infiltrated it into the intercellular spaces of leaves, where it enters in contact with the parenchyme cells. The P^{32} and C^{14} of the virus was found incorporated in intracellular materials in an isotopic ratio identical with the initial virus. However, the normal proteins and free amino acids were not labeled by C^{14}, and unfortunately, the virus did not multiply. Although plant virus systems offer obstacles to isotopic investigation because of the difficulty of penetration by the virus and the failure to produce but a single generation, the initiation of studies with labeled animal viruses should now be possible through use of tissue culture methods.

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METABOLISM OF LIPIDES[1,2,3]

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Digestion and Absorption

Digestion and action of lipase.—Herting & Ames (1) have reported high levels of free fatty acids in stomach contents of rats after feeding fully saturated triglycerides. After feeding hydrogenated lard 49.9 per cent of the total fatty acids recovered from the stomach was in the form of free fatty acids compared to 8.8 per cent when the unhydrogenated fat was fed. It is not clear if this is an effect of a gastric lipase or regurgitated pancreatic lipase nor is it known if different emptying rates of the stomach with the different diets is of importance for this effect. It has to be recalled that the gastric lipase studied by Schönheyder *et al.* (2) had the properties of a tributyrinase with very low activity against glycerides with long chain fatty acids.

Since the demonstration that the monoglyceride formed in the lumen of the small intestine of the rat during digestion is mainly of the 2-configuration (3) interest has continued in elucidating the mechanism of hydrolysis of glycerides by pancreatic lipase. Borgström (4) identified the diglyceride formed by *in vitro* hydrolysis of triolein with rat pancreatic juice as the 1,2-diglyceride. Studies using synthetic model glycerides and labelled acid have also given results indicating that the hydrolysis of triglycerides is directed to the 1,2-diglyceride and the 2-monoglyceride (5, 6). In the work of Schönheyder & Volqvartz (7) the rate of hydrolysis of the tri-, 1,2,- and 1,3-diglyceride and the 1-mono- and 2-monoglyceride of lauric acid by pancreatic lipase was compared. The 1,3- and 1-monoesters were hydrolysed at a higher rate than corresponding esters with a fatty acid in the 2-position. The rate of hydrolysis of trilaurin best fitted with the sequence 1-2-di- and 2-mono-glyceride. It was also observed that the rate of hydrolysis of the 1-monoglyceride decreased with the chain length of the acid; 1-monopropionyl glycerol was only very slowly hydrolysed. The mechanism of formation of 1-monoglyceride, which always makes up part of the monoglyceride fraction obtained during pancreatic lipolysis, is not completely clear. Two routes are possible, i.e., the 1,2-diglyceride is only hydrolysed to the 2-mono-

[1] This review covers literature received in Sweden up to the end of October, 1955.

[2] The following abbreviations are used in this chapter: ATP for adenosinetriphosphate; CF for clearing factor; DPN for diphosphopyridine nucleotide.

[3] This review is limited to absorption and digestion of glycerides, the clearing factor, a subject that does not seem to have been reviewed before, and finally the degradation of cholesterol to bile acids and the metabolism of the latter. The authors are greatly indebted to Drs. S. Lindstedt, A. Norman, and J. Sjövall for generous help in preparing the last mentioned part.

glyceride which then is partly isomerized to the 1-form or the 2-ester bond is also slowly hydrolysed with direct formation of some 1-monoglyceride. The results so far obtained on this subject are not quite conclusive (5, 6), but it has been suggested that the pancreatic lipase is specific for esterified primary hydroxyl groups (5). In contrast to the result obtained for pancreatic lipase it has been claimed that oat lipase attacks only the fatty acids in the 2-position of tributyrin (8).

Harris *et al.* (9) found 4 to 11 per cent monoglyceride in lipides recovered from the lumen of the small intestine of human subjects after feeding fat; of this monoglyceride fraction 50 to 65 per cent was of the 2-form. Blankenhorn & Ahrens (10) made a complete analysis by counter-current distribution of the digestion products of triglycerides recovered from the lumen of the small intestine with a new sampling technique which makes possible the collection of the intestinal contents at any level of the gut. They reported the following lipide composition of the intestinal contents taken below the ligament of Trietz from two human beings after feeding a formula containing triolein: monoglycerides 13 and 17 per cent, diglycerides 9 and 6 per cent, triglycerides 4 and 6 per cent, and free fatty acids 58 and 60 per cent, indicating a rather extensive hydrolysis of the triglycerides before absorption.

Mattson *et al.* (11) reported that the quantity and isomeric forms of the monoglycerides found in the small intestinal content of the rat were similar, regardless of whether dietary glyceride fatty acids had 18 or 10 carbons. The composition of the intestinal contents was only very slightly affected by the addition of 1- and 2-monoglycerides in amounts up to 30 per cent of the triglyceride fed. Increasing the amount of free fatty acids of the fed triglyceride over a certain percentage resulted in a marked rise in the free fatty acid content of the lipides in the intestinal contents, indicating an upper limit of the rate of absorption of free fatty acids as was also found earlier (12). Borgström has reported results to demonstrate the magnitude of the exchange reaction taking place between free fatty acids and glyceride fatty acids occurring *in vitro* (6) and in the lumen of the small intestine of the rat during digestion of triglycerides (18). From these results it can be calculated that at least 20 per cent of the fatty acids of the fed glyceride had been exchanged during digestion in the intestinal lumen. This exchange reaction starts the randomisation of the glyceride fatty acids of fed glycerides which is continued in the intestinal cells resulting in a complete randomisation of fed acids between the 1 and 3 position of the triglycerides of the lymph in the rat (13).

Garton & Duncan (14) fed a mixture of lard and codliver oil to pigs and identified by crystallisation from the depot fat typical pig fat (lard) and codliver oil in oxidized form indicating that part of the glycerides fed had been absorbed in the unhydrolysed form.

Reiser & Williams (15) fed doubly labelled palmitoxyhydroxyacetone to rats and found labelled triglycerides but no unchanged dihydroxyacetone esters in the intestinal lymph. From this result they suggested that esteri-

fication of dihydroxyacetone with fatty acids and subsequent reduction and esterification may be the normal mechanism of triglyceride synthesis during fat absorption. The results, however, do not prove that dihydroxyacetone instead of glycerol is the precursor of glyceride glycerol as has been suggested (16). Monoglyceride is just as well converted to triglyceride during fat absorption as is the hydroxyacetone ester, and it has not been demonstrated that free dihydroxyacetone is incorporated into lymph glycerides to a higher degree than free glycerol.

Feeding of monoglycerides and diglycerides to rats results in triglycerides as the only glyceride in intestinal lymph (15, 17).

Clément & Clément (19) found that saturated fatty acids were preferentially liberated from different vegetable and animal triglycerides by pancreatic lipase and that the iodine number of the free fatty acids increased with the degree of hydrolysis. If this is an effect of a specificity of the lipase for fatty acids of different saturation or if it is attributable to an uneven distribution of saturated and unsaturated fatty acids between the 1,3 and 2 position of the glycerides remains to be elucidated.

Autoxidation of oils has been found to retard the rate of hydrolysis by pancreatic lipase (20). Glyceride ester bonds with α-dimethyl-stearic acid have been found to be very resistant to the action of pancreatic lipase (21). This probably is an effect of the steric hindrance of the two methyl groups.

The effect of bile acids on the rate of hydrolysis of triglycerides by pancreatic lipase has been studied by several workers (5, 22 to 25). The results, however, are contradictory; the differences might be attributable to factors such as the purity of the bile acids used, the substrate and substrate concentrations, buffers, etc. Obviously more detailed studies are needed in this field.

Minard (22) studied the effect of Tweens on the action of pancreatic lipase *in vitro* on corn oil triglycerides. They inhibited the action of lipase; approximately 50 per cent inhibition was obtained at a concentration of 0.05 per cent Tween 80. Bile acids were found to reverse the inhibition. As these experiments were performed at a pH of 8.5, it is difficult to judge the importance of Tweens inhibition on feeding Tweens stabilized fat emulsions. Tweens, however, have not been found to impair fat absorption (26, 27).

Wills (23, 24) has studied the effect of different detergents on the action of pancreatic lipase. In general the anionic detergents tested inhibited pancreatic lipase at higher concentrations and were without effect at lower concentrations. Cationic detergents at low concentrations generally were found to stimulate the action of pancreatic lipase. The increased rate of hydrolysis produced by these detergents and that found for bile salts were not additive.

Mattson *et al.* (5) have reported on the effect of electrolytes concentration on the rate of hydrolysis of triglycerides with pancreatic lipase, a maximal effect being obtained at a molar concentration of about 1.

Addition of cholesterol to triolein in a concentration of 10 mg./ml. has been

found to increase the rate of hydrolysis of the triolein by pancreatic lipase (24). The presence of a water soluble activator of pancreatic lipase has been reported present in gallbladder endothelium (28). Desnuelle *et al.* (29) have reported on the nonactivity of pancreatic lipase in ether solution in contrast to what has been found for lecithinase A (30).

Methods for the assay of pancreatic lipase based on the rate of clearing of a fat emulsion (31) and of potentiometric titration of finely emulsified fat (32, 33) have been published. Balzer *et al.* (34) have modified the method of Willstätter to be used for determination of pancreatic lipase in duodenal content and have given normal values for lipase in human duodenal content (35).

Bernhard *et al.* (36) found anti-autoxidant effect of bile pigment *in vitro* and suggested that these compounds serve as anti-oxidants protecting easily oxidizable substances from destruction in the intestinal tract.

Absorption of fatty acids and glycerides.—While the transport route for saturated fatty acids after absorption has been well established, until recently some uncertainty has existed about the unsaturated fatty acids. It has now been definitely proven that 1–C^{14}–oleic and linolenic acids (37, 38) are absorbed according to the same pattern as the long chain saturated acids, irrespective of the form in which they are fed.

Bergström *et al.* (39) have studied fat absorption on thoracic duct cannulated unanesthetized cats. The results differ from those obtained earlier on anesthetized cats, indicating the profound influence of anesthesia on the absorption process.

Detailed studies of fat absorption in a child with chylothorax have given results in accord with those obtained in animal experiments (40). In these experiments (14, 42) a comparison was made between the component fatty acids of dietary fat and chyle fat. In the case of the long chain fatty acids the chyle and dietary fat resembled each other in their composition with the exception for a lower content of stearic acid and possibly longer chain fatty acids in the chyle as a result of less complete absorption of these acids. The saturated fatty acids with 4, 6, 8, and 10 carbons are practically absent from the chyle fat even when fed incorporated into glycerides. The small amount of decanoic acid that is transported via the chyle in the rat has been found there in the ester form (43), while the main part of the decanoic acid transported via the portal vein after absorption is in the form of the free acid (44). In depancreatized dogs the intestinal absorption of methyl esters of saturated fatty acids is more impaired than the unsaturated and the less affected the shorter the chain length of the acid (45). The absorption and metabolism of α-dimethyl-stearic acid labelled in the 1 position has been studied by Bersgtröm *et al.* (21). The absorbed acid was transported via the lymphatics incorporated into glycerides and phospholipides. While glyceride ester bonds with α-dimethyl-stearic acid are very resistant to the action of the pancreatic lipase, the intracellular enzymes synthesizing glycerides and phospholipides are not hindered by the two α-dimethyl groups.

The digestibility of long chain saturated fatty acids in the rat has been found to be influenced by the form in which the acid is ingested, the monoglycerol ester is the easiest digested (46). It is also influenced by the presence of other fatty acids or fats (47).

Burr *et al.* (48) have compared chylomicron counts and radioactivity in blood after feeding rats C^{14}-labelled fatty acids and report a close parallel between them and, therefore, conclude that the chylomicron count can be used to study variation of absorption of fat. The presence of clearing factor in blood during fat absorption (101, 103) and the perceptible disappearance of chylomicra in postheparin plasma under dark field illumination observed by Swank (49) has to be considered in chylomicron counting.

Singer *et al.* (50, 51) have reported that oleic acid fed to dogs gives rise to chylomicronemia; the same result was also obtained when oleic acid was introduced into Thiry jejunal fistulas in dogs. They consider their results incompatible with the hypothesis of Frazer, a view not shared by Frazer himself (52).

Sahasrabudhe (53) observed differences in the chylomicron response in human beings after ingestion of fat with different contents of high melting fat. The peak in the chylomicrograph is shifted in blends which contain high melting point fractions, indicating retention of fat in the blood over longer periods.

Tasker (54) has studied the effect of choline on fat absorption in rats by studying the quantity and composition of intestinal lymph and could find no evidence that choline significantly influenced the absorption of olive oil as had been stated earlier (55). Similarly Word *et al.* (56) found that severe and prolonged choline deficiency in the rat did not effect the rate of fat absorption. A 20 per cent drop in absorption of fat produced by adrenalectomy in the rat was increased to normal after administration of cortisone and deoxycorticosterone (57).

The effect on fat absorption of excluding the bile from the small intestine has been studied in the rat (58, 59, 60) and in the dog (61). Bernhard & Ritzel (58) fed biosynthetically deuterium labelled fat to rats with bile fistulae and found an absorption of 7 to 21 per cent. The deuterium excess in fecal lipides was 62 to 75 per cent of that of the fed lipides, indicating a dilution by inactive fatty acids which is believed to be derived from an increased excretion of fat to the gut in the absence of bile. Borgström (59) in similar experiments fed corn oil containing a small amount of C^{14}-labelled free palmitic acid to rats and found an absorption amounting to a mean of 65 per cent when calculated from activity data which probably are not representative for all the fat fed. A striking difference between normal and bile fistula rats was the low concentration of lymph lipides in the latter, both in fasting and during fat absorption. Only about one fourth of the absorbed activity was found transported via the lymphatic pathway.

Kim & Bollman (60) similarily reported that the content of fatty acids in intestinal lymph from rats with biliary fistula decreased to about 1/15 of

that of normal rats. They could not demonstrate any absorption of corn oil in the absence of bile and pancreatic juice in the rat. The free fatty acid of corn oil and oleic acid were absorbed in small amounts.

In dogs Pessoa *et al.* (61) found a considerable absorption of corn oil (70 per cent) and free fatty acids (55 to 75 per cent) in the absence of bile or pancreatic juice. Low melting point fat is better absorbed. The so-called endogenous lipide excretion was found to be increased about three times in biliary fistula and pancreatic duct ligated dogs. This paper also contains data to show that fecal phospolipides in biliary-fistula dogs increase after feeding unsaturated fat but is low after feeding a more saturated fat. The implication of these findings is not clear. Pihl (62) observed an increase in fecal phospholipides in rats which were fed free fatty acids in agreement with earlier observations (63). Impaired fat absorption has been reported in dogs lacking pancreatic juice (64, 65).

The question of origin of the so-called endogeneous fat in feces has been debated and different opinions expressed. Holasek has arrived at the conclusion that the fecal lipides in the normal rat (66) and in the normal human being (67) are derived from nonabsorbed food fat and from bacterial fat. The increased fat excretion in rats with bile fistula is to be ascribed to increased amounts of bacteria in the feces and not to any fat excretion (68) by the gut as believed by others (58).

Wollaeger *et al.* (69) studied the fecal lipides in two normal human subjects on different diets and concluded that a large portion of the fecal lipides on a general mixed diet is derived from unabsorbed dietary fat. The administration of a large amount of triolein led to excessive amounts of fecal lipides, indicating a difference in the efficiency of absorption of this fat.

Norcia & Lundberg (70) in studies on rats fed simple triglycerides found that their presence in the diet was without effect on the composition and quantity of excreted nondietary fat nor did the nondietary fat change with a change in the composition of the body fat brought about by long-term ingestion of different fat. The conclusion drawn by these authors is in accord with that of Holasek (66, 67, 68), i.e., endogenous fecal fat is derived from intestinal bacteria.

Studying fat absorption with C^{13}-labelled oleic acid Blomstrand (71) concluded that in the normal human subject only about 1 gm. of fecal fatty acids per day is of nondietary origin. This is in accord with the results of Lewis & Partin (72) that normal human subjects on an essentially fat free diet excrete about 2 gm. of ether soluble lipide per day. Work by Blomstrand (71) and Blomstrand *et al.* (73, 74) with labelled fatty acids indicate that in the case of nontropical sprue, congenital bile duct atresia, and fibrosis of the pancreas in human beings, the major part of the fecal lipides are derived from unabsorbed dietary fatty acids combined with a moderate increase of endogenous fat. No indication of a large secretion of fat to the intestine could be found.

Singer *et al.* (51) have reported on the presence of particles similar in

appearance to chylomicrons in the lumen of jejunal Thiry loops of fasting dogs. They were named enterolipomicrons and were supposed to represent lipides secreted in the intestinal lumen. No chemical analysis of the particles, however, was reported.

Kremen *et al.* (75) reported that the proximal 50 to 70 per cent of the small intestine in dogs could be removed without any impairment of fat and protein absorption. The distal part of the small intestine was, however, found to be of profound importance for fat absorption.

Absorption of phospholipides.—Lecithinase activity has been reported in rat pancreatic juice (76); the results are based on the disappearance of lipide-phosphorous and give no indication of the nature of the lecithinase present.

Bloom *et al.* (77) and Blomstrand (78) have demonstrated that after feeding phospholipides biosynthesized with labelled fatty acids a significantly greater amount of activity was obtained in the phospholipide fraction of the thoracic duct lymph of rats than after feeding the same acid as glyceride or free fatty acid. These results indicate that a significant part of the fed phospholipides is absorbed before undergoing complete hydrolysis. Blomstrand (79) has also shown that a significant part of the phospholipide glycerol appears in the thoracic duct lymph as phospholipide glycerol. Buensod *et al.* (80) have reported that neither deuterium labelled free glycerol nor glycerol-phosphate are important precursors of phospholipides in the intestinal mucosa during fat absorption.

Blomstrand (81), studying the absorption of phospholipides containing labelled palmitic acid in bile-fistula rats, found an absorption of about 68 per cent, and only about one third of the absorbed activity was transported via the thoracic duct lymph as compared to the normal condition.

The literature on the role of the phospholipides of the intestinal mucosa in fat absorption was treated in this series last year by Zilversmit (82).

Absorption of hydrocarbons.—Daniel *et al.* (83) studied the absorption of liquid paraffin fed to rats. From long-term balance studies it appeared that the absorption of the paraffin was enhanced by the presence of olive oil. In short term experiments an absorption of about 60 per cent of liquid paraffin dissolved in olive oil (50 per cent) was demonstrated. The unsaponifiable fraction of the intestinal lymph after feeding olive oil-liquid paraffin was increased over that when olive oil was fed alone. These results are in accord with earlier extensive work on hydrocarbon absorption by Bernhard & Scheitlin (84).

The metabolic activity of the intestinal mucosa during fat absorption has been demonstrated in several papers. After feeding hydrocarbons deuterated in the 1,2 or 2,3 position Bernhard *et al.* (85) recovered the hydrocarbons in the intestinal lymph with lower deuterium content, indicating the dehydrogenation of the hydrocarbons at this site, probably as a step in the oxidation of the hydrocarbons. On the other hand, 9,10-di-deuterostearic acid did not lose any deuterium during absorption. From differences in iodine number of fed fat and fat recovered from the thoracic duct lymph of dogs, Pontremali

& Montini (86) concluded that desaturation of fatty acids took place during the passage of the enteric mucosa.

Blomstrand & Rumpf (87) fed 1-C^{14}-cetyl alcohol to rats and could recover more than 50 per cent of the absorbed activity from thoracic duct lymph; of this only 15 per cent was present as the alcohol, the remainder had been oxidized to palmitic acid.

Changes in intestinal lymph during absorption of lipides. Borgström & Laurell (88) have studied the changes in lymph volume and lymph protein during absorption of fat in the rat. The lymph volume per time unit increases strongly after peroral administration of fat, and the optimal effect was found to occur before the optimum for lymph lipides. The protein concentration of the lymph stays practically constant in spite of the increased lymph volume in contrast to what is seen when saline or glucose is fed. The lipoprotein of the type found in plasma was not found to increase in lymph during fat absorption. Similar results concerning the lymph flow, lymph fat, and protein have also been reported by Tasker (54). The ratio between phospholipide and total fat in the lymph during fat absorption is not constant, the total lipides rise and fall more rapidly than do the phospholipides (54). A probable explanation is that a significant part of the lymph phospholipides is derived from plasma phospholipide (89).

Laurell (90) has isolated chylomicrons from rat lymph by flocculation with toluidine blue and found them to be composed of 8.5 per cent phospholipides, 2 per cent protein, and 0.3 per cent carbohydrate in addition to neutral fat that makes up the remainder. Similar composition was also reported for human chylomicrons (230). Robinson (231) after isolating chylomicrons from rat lymph by high speed centrifugation reported them to be free of protein.

Reviews on different aspects of the digestion and absorption of fats have been published by Bergström & Borgström (91, 92), Frazer (93, 94), Reiser (15, 95), and Annegers (96).

The Clearing Factor

As this subject has not been reviewed previously in this publication, we felt it desirable to go back to 1943 when Hahn (97) described the *in vivo* clearing of alimentary hyperlipemia after intravenous injection of heparin, whereas addition of heparin to lipemic plasma *in vitro* was without effect. Andersson & Fawcett (98) later demonstrated that intravenous administration of heparin resulted in the appearance in blood of a "clearing factor" (CF) which was able to clear lipemic plasma *in vitro*.

Mode of production of CF.—CF was first demonstrated in blood after intravenous injection of heparin. Low endogenous CF has later been reported in rats (99) and in man (100). Jeffries (101) was unable to demonstrate any CF in rat blood with the rats on a fat free diet but could demonstrate a transient CF in rat plasma during absorption of fat. Hood *et al.* (102) could not demonstrate any CF activity in postalimentary lipemic human

plasma at room temperature. However, Robinson *et al* (103) found that alimentary lipemic human plasma incubated *in vitro* at 37°C. showed some CF activity. Engelberg (104) found endogenous CF in the plasma of 25 out of 39 humans while serum of these plasmas usually did not show any CF. Blood CF has been found *in vivo* in dogs during peptone and anaphylactic shock (105). Several other substances have also been found to produce clearing activity in blood *in vivo*, i.e., Treburon and Paritol (106), glycogen and gum arabic (107), phosphomolybdate, phosphotungstate and silicotungstate (108), and dextran sulphate (109), indicating that the heparin is not specific for the formation of CF.

Anfinsen *et al.* (99) demonstrated three essential factors for the formation of the CF-tissue extract, heparin, and plasma. The cofactor supplied by normal plasma was termed coprotein. The coprotein was later found to be composed of at least two fractions, albumin and another unknown factor (110). The role played by albumin was considered to be an acceptor for the fatty acids released by the CF (111). Similar views were also expressed by several other workers (100, 112, 113). Korn (114), later working with CF from heart, found no specific requirement for albumin; calcium ions, for example, are at least as good a fatty acid acceptor as albumin (115). Seifter & Baedes (116) working with postheparin plasma CF found that both calcium and albumin were necessary to restore maximal rate of clearing of chylomicrons suspended in saline solution. The coprotein of Gordon *et al.* (111) probably is identical with the α- and β-lipoproteins of serum (110, 115).

Sites of production of CF.—Perfusion of isolated organs of rat and dog with heparinized plasma or blood indicated that the abdominal viscera and thoracic regions were capable of producing CF (99). Jeffries (117) similarly obtained CF from perfusions of lung and abdominal viscera with the exception of the liver which instead was found to destroy CF. He also obtained CF from perfused hind limbs of rats in accord with earlier results of Weld (118, 119) but contrary to Anfinsen *et al.* (99). Extracts with CF activity have also been obtained from different organs of which lung and heart were reported most active (99). Korn (114) further studied the distribution of CF and found activity in beef and pig heart but could demonstrate no appreciable quantities of CF activity in any other tissue. Later, however, Korn (120) obtained CF activity in aqueous extracts of adipose tissue. The properties of the CF active factor in extracts from heart and adipose tissue and of serum are so similar that they are believed to be identical. The effect of injected heparin and other substances might therefore be an overflow of the CF into the blood where it normally is found only in insignificant amounts. It is not known at present if heparin is the natural factor for release of CF to the blood.

Effects of the CF.—It was early recognized that fatty acids are liberated during the clearing reaction *in vitro* (110, 112, 121 to 126) and that a partial hydrolysis of the triglycerides of the chylomicrons occurred (122). It was also found that all lipides that left the turbid form during the clearing reaction

in vitro could be recovered as nonesterified fatty acids bound to albumin (112). Grossman *et al.* (113, 125) reported that injection of heparin into fasted rats gave a moderate increase and in lipemic rats a marked increase in free fatty acid content of plasma. They also observed a moderate increase in free fatty acids in plasma in association with alimentary lipemia without previous heparin injection. They calculated that all the fatty acids released could be bound to the albumin of rat blood. Robinson *et al.* (103) reported an increase in free fatty acids of human plasma after consumption of a fat meal. McDaniel & Grossman (128) incubated C^{14}-labelled fat emulsion with postheparin plasma and could demonstrate by paper electrophoresis that part of the radioactivity migrated with the albumin fraction.

During clearing of plasma *in vitro* an increase in α-lipoprotein and a concomitant decrease in certain low density lipoproteins have been reported (127). Similar interpretations have been also made from *in vivo* studies by Graham *et al.* (129) and Nikilä (100). These changes were thought to be partly at least caused by a transfer of lipide from β- to α-lipoprotein. Herbst *et al.* (130), however, have criticized the interpretations of the above-cited authors and could find no evidence to support the concept that injection of heparin caused a transformation of β- to α-lipoprotein. They think that what these authors have believed to be α-lipoprotein is in fact the β-lipoprotein fraction. Both lipoprotein fractions show an increased mobility after heparin injection which is probably caused by binding of fatty acids to the lipoproteins.

Laurell (131) and Gordon (132) have shown that the increased mobility of the plasma lipoproteins during the clearing reaction can be obtained if free oleic acid is added to normal serum and that both these reactions could be reversed by adding serum albumin. It thus is possible that the changes in the serum lipoprotein pattern observed during the clearing reaction, partly at least, are secondary to the liberation of free fatty acids from low density lipoproteins by the action of the CF.

Properties of the CF from blood.—Nikkilä (100) precipitated the CF of postheparin human serum at low ionic strength and obtained a twenty-twofold purification. Purification with salt precipitation is not possible as a result of inactivation of the CF. Nikilä (100) also localized the CF of post-heparin plasma by zone-electrophoresis in the β-globulin fraction. Similar results were reported by Graham *et al.* (129) from ultracentrifugation studies and by Anfinsen *et al.* from alcohol fractionations (99). Seifter reported the use of the ion-exchange resin IR-120 for the purification of the CF from post-heparin plasma (116). Hood *et al.* (102) found CF to be absorbed on asbestos and $Mg(OH)_2$.

Detailed studies of the properties of post-heparin plasma CF have been published by Brown *et al.* (110), Spitzer (103), Levy & Swank (106), and Meng *et al.* (134). Robinson *et al.* (135) compared the CF activity of post-heparin plasma and of postabsorptive plasma from rats and found identical behavior to factors such as temperature, pH, and inhibitors.

Properties of the CF from heart and adipose tissue.—The clearing activity

of extracts of heart and adipose tissue has been studied thoroughly by Korn (114, 115, 120, 136). A most important question in this connection is that of the identity of the CF of postheparin plasma and the factor obtained by Korn from heart and adipose tissue. They both show the same behavior to salt, heparin, and protamine. More data, however, at present seem to be at hand for the factor from heart and adipose tissue than from postheparin CF. The factor from heart has a requirement for certain cations, and heparin activation cannot be demonstrated in the absence of cations. While the hydrolysis of chylomicrons and lipemic plasma is catalysed by heart extracts, a fat emulsion is not attacked. After addition of normal serum or preincubation with serum such activated emulsions are hydrolyzed as rapidly as are chylomicrons (114). A study of this activation indicated that the α- and β-lipoproteins of serum were the factors responsible for the activation of the oil emulsion (114). The factor obtained from adipose tissue differs from that from heart in so far that no heparin or cations are necessary for activity (120).

It seems that data at hand indicate a close relationship between the CF of postheparin and postabsorptive plasma and the factor from heart, but the demonstration of their identity is not yet based on conclusive evidence. The low clearing activity of postalimentary plasma, however, seems to throw some doubt on the idea that the clearing of postalimentary blood *in vivo* is caused by the clearing factor released to the blood. Another possibility is that the lipemic blood *in vivo* is cleared during the passage of the capillaries of certain tissues, i.e., heart and adipose tissue, and that the presence of clearing activity in blood is attributable to an overflow of clearing factor into the blood.

CF in relation to plasma lipase and ali-esterase activity.—Injection of heparin has not been found to increase significantly serum tributyrinase activity (137, 138) or plasma esterase activity, assayed with β-naphthol-laurate as substrate (110). Studies with different inhibitors and activators further have shown the nonidentity of the CF and normal blood tributyrinase or pancreatic lipase (115, 134, 138, 139).

Substrate for assay of CF.—In the early development lipemic plasma (98 to 100) and chyle (140) were the substrates most frequently used. Later oil emulsions were used by several workers for assaying plasma CF (99, 110, 125, 134). An oil emulsion activated with normal plasma and albumin has been reported to give a clearing more linear with the CF activity than earlier substrates (141). In assaying CF activity of organ extract, artificial oil emulsions have to be activated by addition of serum.

Intermediary Metabolism of Bile Acids

Methods for the separation and determination of bile acids.—Recent progress in the study of bile acid metabolism depends largely on the elaboration of new methods for the separation and determination of the bile acids. It thus seems necessary to summarize the more important developments in this field.

Ahrens & Craig used counter-current distribution for the analysis of bile acids in ox bile (142). Bergström & Sjövall have separated bile acids using reversed phase partition chromatography (143), and this method has been further developed by Sjövall (144) and Norman (145). Partition chromatography of bile acids has also been described by Mosbach *et al.* (146). Separation of bile acids with paper chromatography has been described by Kritchevsky & Kirk (147), Sjövall (148 to 151), Haslewood (152), and others (153 to 156). Adsorption chromatography of the methyl and ethyl esters of bile acids on alumina has been used by Haslewood & Wootton (157), and Wootton (158, 159) has determined the bile acids in human bile with infrared spectroscopy after separation on silicic acid. This method has been further extended by Wiggins (160). Arima (161) has reported the separation of free and conjugated bile acids on alumina.

New methods for the quantitative determination of bile acids have appeared. Isaksson (162, 163) has determined chenodeoxycholic acid with a modified Liebermann-Burchard reaction. The chromogens formed when bile acids are dissolved in sulfuric acid have been studied by Kier (164), Mosbach *et al.* (165), and Eriksson & Sjövall (166, 167). These chromogens have been used by Sjövall (168) in combination with paper chromatography for the determination of bile acids in synthetic mixtures and in bile.

Sulfuric acid chromogens, fluorescence in sulfuric acid, and several colorimetric methods for the direct determination of bile acids in biological material are still very commonly used, but great care should be taken in interpreting the results because of the sensitivity of all such methods to interfering material.

The conversion of cholesterol to bile acids.—The now classical observation by Bloch, Berg & Rittenberg (169) that deuterated cholesterol was transformed into cholic acid in the dog has been repeatedly verified for different animals during recent years. Bergström (170) isolated taurocholic acid as the main end product of cholesterol metabolism in the rat. Siperstein and coworkers (171) fractionated the radioactive metabolites found in bile and feces of rats fed cholesterol-4-C^{14}. Most of the activity in the bile as well as in the feces was in the acidic fraction, presumably as bile acids. For further identification of these acids Siperstein *et al.* (156) used a paper chromatographic method combined with direct crystallization with carrier. In a bile sample obtained soon after the injection of cholesterol they found 2 to 5 per cent of the activity as lithocholic acid. While in the early samples most of the activity was present as chenodeoxycholic acid and only a small amount as cholic acid, the reverse was true for later samples. It was suggested that chenodeoxycholic acid might be an intermediate in the conversion of cholesterol to cholic acid, an assumption that appears less likely in this species in the light of other investigations. Bergström & Norman (172) using column chromatographic techniques found taurine-conjugated cholic and chenodeoxycholic acid to be the main labelled products in the bile of rats injected intraperitoneally with cholesterol-4-C^{14}. Chromatography of the fecal acids

showed the activity to be distributed over a variety of compounds probably arising from the action of microorganisms. Ekdahl & Sjövall (173) have undertaken a study of the metabolic products of cholesterol in the rabbit, an animal that has been extensively used for studies of the hypercholesterolemic states. They found glycine conjugated deoxycholic acid and a small amount of cholic acid in the bile and again a large number of other acidic products in the feces as cholesterol metabolites.

The conversion of cholesterol to bile acids in man has been reported by Rosenfeld, Hellman & Gallagher (174) and Siperstein & Murray (175). The latter authors separated the bile acids with paper chromatography and found most of the activity in the glycocholic acid spot. Cholic and "deoxycholic" acid was identified by crystallization with carrier.

Bile acids from acetate.—Zabin & Barker (176) have administered biosynthetically labelled cholesterol to rats. The distribution of the activity between the carboxyl carbon and the rest of the molecule of the cholic acid which was isolated was in accordance with the assumption that no more than three carbon atoms had been removed in the conversion. When 2-C^{14} acetate was given, the same distribution was found, indicating that the cholesterol had been an intermediate in the formation of cholic acid. Staple & Gurin (177) gave methyl- and carboxyl-labelled acetate and isolated the carboxyl-carbon from cholic acid, which was found to arise from the methyl-carbon of acetate. In most cases the specific activity was found to be higher than that of bile cholesterol during the experimental period (three days). Various explanations for this fact are discussed. Dayton and co-workers, (178) found a higher specific activity in bile acids than in bile cholesterol only during the first day after the administration of acetate (cf. 174). Thus at the present time it remains an open question if cholesterol is an obligatory intermediate in the formation of bile acids or not.

Friedman, Byers & Gunning (179) have injected rats with hypercholesterolemic serum and reported 60 per cent increase in cholate excretion. The hypercholesterolemic serum was obtained from bile duct ligated animals which had been fed large amounts of cholic acid. It has been pointed out by Magee (180) that the excess cholate might originate from the transfused blood. Magee (180), using serum from thyroidectomized dogs fed cholesterol, observed no rise in cholate excretion when it was transfused into bile fistula dogs.

Mechanism of conversion of cholesterol to bile acids.—In the search for possible intermediates in the conversion of cholesterol to bile acids Bergström & Pääbo have injected Δ^4-cholestone, Δ^5-cholestenone, 3(β)hydroxycholestane, 3(α)hydroxycoprostane, and epicholesterol into bile fistula rats. All the compounds gave rise to acidic products, none of which was identical with cholic acid. The results have been summarized by Bergström (181). In an attempt to decide whether the primary step is a hydroxylation of the steroid nucleus or a degradation of the side chain Bergström and co-workers (181, 182) have injected 3(α)7(α)12(α)trihydroxycoprostane, and 3(β)Δ^5

cholenic acid prepared from hyodeoxycholic acid-24-C^{14} (183) which would appear as intermediates in the extreme cases; the former when the hydroxylation was completed first and the latter when the side chain was completely degraded first. Whereas $3(\alpha)7(\alpha)12(\alpha)$trihydroxycoprostane rapidly gave rise to cholic acid, this was not the case with the cholenic acid. The results thus indicate that the hydroxylation is more or less completed before the degradation of the side chain is finished. The observation that chenodeoxycholic acid is not an intermediate (197) in the rat is also in accordance with this evidence.

Harold, Jayko & Chaikoff (184) have reported that epicholesterol gives rise to bile acids which are not identical with cholic or chenodeoxycholic acid. An *in vitro* system for the side chain degradation of cholesterol has been reported by Anfinsen & Horning (185).

Factors influencing the conversion of cholesterol to bile acids.—Thompson & Vars (186, 187) have followed the excretion of cholic acid and cholesterol in bile fistula rats and in those with altered thyroid function. In the untreated animal there was a rapid fall in the cholic acid excretion on the first day after operation followed by a rise to a plateau level of about 35 mg. per day reached in about four days. As rat bile furthermore contains chenodeoxycholic acid, the total amount of bile acids formed per day can correspond to more cholesterol than the amount present in the liver. The authors consider the high plateau value to represent the normal amount of cholic acid formed in an intact animal and ascribe the low values during the first days to deranged hepatic function. It rather appears to us to represent the maximal capacity of the liver, 5 to 10 times larger than the normal syntheses, that is reached when there is no enterohepatic circulation that presumably has a regulatory influence on the rate of formation of bile acids. Rats fed thyroid showed a marked decrease in cholic acid and increase in cholesterol excretion. Thiouracil treated rats excreted less cholic acid and cholesterol than control animals, though the differences were smaller than in the hyperthyroid group.

Rosenman, Friedman & Byers (188, 189, 190) have also published papers on the same subject. They conclude that the metabolism of cholesterol is increased in hyper- and decreased in hypothyroid states. Their results are, however, based on the excretion in bile fistulas during the first day after operation and should be judged in the light of investigations cited above. Weiss & Marx (191) have injected mice in the hyper- and hypothyroid states with labelled cholesterol and followed the distribution of activity between the acid and neutral sterol fractions in carcass and excreta. Their results indicate that the thyroid hormone stimulates the conversion of cholesterol to acidic products as well as the total excretion of labelled products.

Interconversion of hydroxy- and ketocholanic acids.—Previous work on the transformation of hydroxy- and ketocholanic acids has mostly been carried out by Japanese workers and led to the view that less hydroxylated acids arise from cholic acid via ketocholanic acids. Most of this work has been

summarized in an review by Haslewood (192), who pointed out that the methods used by these workers are subject to criticism. A few papers concerning the metabolism of dehydrocholic acid in the animal organism have appeared in the last few years (193, 194).

Through the synthesis of C^{14}-labelled bile acids by Bergström, Rottenberg & Voltz (195) and the development of chromatographic methods for the separation of bile acids it has become possible to study the problems of interconversion of bile acids *in vivo* and *in vitro* more closely (for recent reviews see 181, 196). Most experimental work has been done on rats. The bile acids of the rat are almost totally conjugated with taurine and consist mainly of cholic and chenodeoxycholic acid (170, 197) and traces of lithocholic acid (156).

After intraperitoneal injection of labelled cholic acid to a bile fistula rat the only radioactive compound that could be recovered in the bile after hydrolysis was cholic acid (198). Byers & Biggs (199) injected tritium labelled cholic acid into a bile duct ligated rat and were unable to isolate any radioactive cholesterol. On the basis of these experiments cholic acid might be regarded as a metabolic end product at least in the rat. The bile acids with fewer hydroxyl groups are metabolized in different ways. Bergström, Rottenberg & Sjövall (198) found that deoxycholic acid was rapidly converted to cholic acid in the bile fistula rat. This result was confirmed by Matschiner and co-workers (200). In the rabbit, however, Ekdahl & Sjövall (201) showed that intravenously injected labelled deoxycholic acid was not hydroxylated but only conjugated and excreted in the bile. In this animal, deoxycholic acid is the main bile acid.

Chenodeoxycholic acid that occurs normally in rat bile is not converted into cholic acid but is converted into two compounds which on partition chromatography behave similarly to cholic acid, none of them, however, identical with this acid (197). Mahowald, Elliott & Thayer (202) confirm this, having made a preliminary communication on the crystallization of these two acids from rat bile. The structure of the acids is as yet unknown.

The metabolism of another labelled dihydroxycholanic acid, hyodeoxycholic acid ($3\alpha,6\alpha$-dihydroxy), has been investigated by Matschiner, Hsia & Doisy (203). They found a 5 per cent conversion to an acid which moved similar to cholic acid in the chromatogram. It was neither identical with cholic acid nor with the hyocholic acid recently isolated from hog bile by Haslewood (204).

Bergström *et al.* (205) have also studied the metabolism of less hydroxylated bile acids in the rat. Lithocholic acid when injected intraperitoneally was rapidly excreted in the bile and transformed into at least three compounds more polar than lithocholic acid but none identical with cholic acid. Unsubstituted cholanic acid is rapidly and almost completely converted to a large number of more polar compounds (181).

The 7α-hydroxylation of deoxycholic acid has been studied *in vitro* by Bergström & Gloor (206 to 211) using rat liver slices and homogenates.

From taurodeoxycholic acid practically pure taurocholic acid is found in slices and homogenates (207, 208). Mitochondria or microsomes alone or with ATP do not hydroxylate, whereas the best yield of taurocholic acid is obtained with microsomes plus supernatant and pure ATP. Impure ATP preparations contain a factor that with particle free supernatant yields more polar products, only a minor part of which is cholic acid (211). Not only structural factors of the substrate are thus of importance for the specific hydroxylations, but other factors might influence the course of the hydroxylation.

The intestinal absorption of labelled bile acids in fistula rats has been studied by Norman (212), Portman & Mann (213), and Sjövall & Åkesson (214). Taurocholic, glycocholic, and glycodeoxycholic acids were rapidly absorbed and excreted with about the same rate, i.e., 80 to 90 per cent of the isotope appeared in the bile in 2 hr. following oral administration. This rapid absorption and re-excretion of the bile acids indicate that the time usually given in literature (about 8 hr.) for one enterohepatic cycle of the bile acids is far too long, at least in the rat which does not have a gallbladder. In a study of the enterohepatic circulation of carbon 4 of cholesterol in the rat, Siperstein, Hernandez & Chaikoff (215) found a rapid intestinal absorption and excretion of the products formed.

In lymph fistula rats only traces of isotope have been found in the lymph after the administration of biosynthetic taurocholic acid labelled in the taurine part (213) or in the cholic acid part (214). These observations confirm many older findings that bile acids are carried to the liver by the portal blood.

The results on the metabolism of the different cholanic acids described, all have been obtained in fistula animals or *in vitro*. Some experiments with intact rats have also been reported. Bergström, Rottenberg & Sjövall (198) found that 24 hr. after the intraperitoneal administration of labelled cholic acid 85 per cent of the injected radioactivity was present in the feces and intestinal content. Four per cent was found in the liver and 11 per cent in the intestinal wall. Less than 1 per cent was recovered in the expired carbon dioxide.

Matschiner *et al.* (200) found less than 1 per cent of the injected radioactivity in the urine after administration of labelled deoxycholic acid, whereas practically all activity appeared in the urine of bile duct ligated animals. Thus the hydroxylated cholanic acids are not broken down in the animal but normally excreted in the feces (for microbiological transformations see below). Lindstedt & Norman (216), measured the faecal radioactivity after administration of several labelled bile acids and have calculated the half life of bile acids to be about 2.5 days in the rat. One of the factors influencing the turnover of bile acids is the intestinal flora. In rats treated with oxytetracycline (terramycin) and sulfa drugs the half life of bile acids is considerably longer than in normal rats (216).

Conjugation of bile acids in vivo.—With the aid of chromatographic or

countercurrent methods several investigations of the composition of the bile from different animals have been made. In no case has any free bile acids been reported. It is, therefore, probable that free bile acids are not normally excreted (192).

The conjugation of individual bile acids and their metabolites have been investigated in the rat with 24-C^{14}-labelled bile acid (217). Bile acids normally present in rat bile, i.e., cholic and chenodeoxycholic acid, were excreted almost totally conjugated with taurine. Only 1 to 2 per cent of the activity appeared to be glycine conjugated, and no free bile acids could be detected. Similar results were obtained after administration of deoxycholic, lithocholic, and cholanic acids. These findings indicate that different substituents of the cholanic acid molecule do not interfere with the conjugation. After administration of deoxycholic acid-24-C^{14} to rabbits the labelled material in the bile was recovered as glycine conjugates (201).

Interconversion of taurine and glycine conjugates.—The question if any interconversion of conjugated bile acids occurs in the liver is still open. Recently certain experiments have been published that give some information concerning the state of the conjugated bile acids in the enterohepatic circulation. Portman & Mann (213) have shown that most of the isotope (mean = 69.2 per cent) is recovered in bile after administration of taurocholate-S^{35}, indicating that the major part of the taurocholic acid is absorbed and excreted without hydrolysis. Another way to investigate this hydrolysis was to give glycine conjugates to bile fistula rats. When Norman (212) injected glycocholic acid intraperitoneally into bile fistula rats, all the activity in the bile was still present as glycocholic acid. After oral administration, however, 1 to 5 per cent of the labelled cholic acid was present as taurine conjugate. After about 6 hr. of enterohepatic circulation more than 50 per cent of the labelled bile acids were found to be taurine conjugated. These findings indicate that there occurs a certain hydrolysis of the glycine conjugate during the intestinal passage, but the absorbed free bile acids are completely reconjugated with taurine. The enterohepatic circulation thus mainly involves conjugated bile acids.

Conjugation of bile acids in vitro.—The conjugation of cholic acid *in vitro* has been reported to occur in liver tissue from the rat (206, 207, 218) guinea pig (219), and human liver homogenates (209). Bergström & Gloor (207, 208) have shown that after incubation of a tracer dose of 24-C^{14}-labelled cholic, deoxycholic, chenodeoxycholic, or lithocholic acid with rat liver homogenates the major part of the labelled products were present as taurine conjugate, and only a small amount conjugated with glycine. They have further studied the conjugation and 7-α-hydroxylation of deoxycholic acid and obtained some indications that the reaction proceeds via taurodeoxycholic acid to taurocholic acid. With the aid of taurin-S^{35} and glycine-C^{14} bile acid conjugation has been studied by Bremer (220), who found that the taurine is almost quantitatively conjugated in spite of the fact that the glycine content of rat liver is about three times that of taurine. Bergström

& Gloor (209) have found that homogenates from human liver biopsies conjugate cholic acid with glycine to about 60 per cent. The addition of 3 moles of taurine per mole of bile acid, however, changed the composition to 90 per cent taurocholic acid and 10 per cent glycocholic acid whereas addition of glycine did not have such a marked effect. It thus appears that the specificity of the enzyme systems for taurine with respect to glycine as well as the availability of taurine are two major factors for controlling the ratio of glycine and taurine conjugates.

Bremer & Gloor (221, 222) and Siperstein (218) have studied the conjugation of cholic acid in subfractions of rat liver homogenates, and found that the enzyme systems which affect conjugation with taurine and glycine were present in the microsomal and particle free preparations. For optimal enzymatic activity the reaction required the presence of Mg^{++}, nicotinamide, DPN, ATP, and coenzyme A. When coenzyme A and ATP were added the particle free supernatant could be omitted. The conjugation of benzoic acid with glycine to hippuric acid, however, requires the presence of mitochondria but is stimulated by the same agents (220). This reaction has been shown to proceed via benzoyl-coenzyme A as an intermediate. The fact that an analogous reaction is involved in the conjugation of cholic acid is supported by the formation of a hydroxamic acid of cholic acid by liver homogenates, independently described by Bremer (223) and Elliott (219).

Action of microorganisms on bile acids.—Separation of the labelled acidic steroids in rat feces showed that most of the conjugates present in bile had been split and the bile acids further modified (172, 224). In rats Norman (225) has studied the influence of oxytetracycline (terramycin) and a sulfa drug on this reaction in the rat. The labelled material excreted in feces after administration of cholic acid-24-C^{14} was then identified mainly as taurocholic acid with small amounts of glycine conjugate, i.e., in the same form as excreted in the bile. This indicates that the splitting of the peptide bond and further modification of the bile acids are mainly a result of the action of intestinal microorganisms.

Sjövall has chromatographed the content of different parts of the intestinal tract after administration of cholic acid-24-C^{14} to normal rats (181). The transformation was found to appear first when the intestinal content reached the caecum and colon. The bile acids in the entire small intestine were practically identical with those of the bile. These findings indicate that in the rat the peptidases present in the small intestine did not attack the peptide bond of taurocholic acid.

The microbial splitting of conjugated bile acids has been further studied *in vitro* with different intestinal microorganisms. Norman & Grubb (226) found that well known intestinal inhabitants both of the aerobic and anaerobic type, i.e., species of *Clostridia* and enterococci, are capable of hydrolyzing the peptide bond in conjugated bile acids. It is noteworthy that these bacteria are also known to be highly resistant to bile in the media.

As a result of the action of microorganisms the bile acids do not appear

unchanged in the feces, but the products have not been identified. The action of microorganisms on free bile acids has been reported by several authors. Ercoli & Ruggieri showed the reduction by yeast of 3,6-diketocholanic acid to 3-hydroxy-6-ketocholanic acid. The oxidation of cholic acid to 3,12-dihydroxy-7-ketocholanic acid and 3-hydroxy-7,12-diketocholanic acid and the reduction of 3,12-dihydroxy-7-ketocholanic acid has been effected by *Escherichia coli* (227). Hayaishi *et al.* (228) have partially purified an enzyme from *Escherichia freundii* capable of reversibly oxidizing lithocholic acid to 3-ketocholanic acid. The enzyme required the presence of DPN and sulfhydryl groups. A *Nocardia* strain isolated from soil has been found to convert cholic acid (229) into what was supposed to be a 3-keto-4,6-cholenic acid derivative.

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BIOCHEMISTRY OF CELLULAR PARTICLES[1,2]

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Introduction

Studies of subcellular particulates have become so numerous and so diverse that it is not possible to cover all the ramifications of this field of cytochemistry in a restricted review article. The subject matter of this review will consequently be limited mainly to selected cytochemical topics and their relation to biochemistry in general. The reader is referred to other reviews on cellular particles (1 to 18) for further information.

The fact that cells, as seen under the microscope, are exceedingly complex structures, and not simply homogeneous masses of protoplasm, at once implies that this arrangement must have some functional significance. For if all compounds and enzymes were distributed uniformly throughout the cell, there would be no reason for the discrete subcellular organization we know the cell to have. It is consequently of great importance to ascertain both the chemical composition and the enzymatic functions of each subcellular structure and to determine the interactions that must occur between various cell organelles. Only in this manner can we hope to arrive at a true understanding of how cells function and why the cells of various tissues differ.

At the moment, studies of subcellular structures are still in their infancy. There is considerable confusion and controversy about the results obtained and the methods to be used. However, the isolation of subcellular particulates from tissue homogenates has emerged as a powerful tool for the study of subcellular function. Although this method, the so-called cell fractionation technique, has a number of disadvantages that a priori would seem to disqualify it (cf. 2), a number of results obtained in cell fractionation experiments have been confirmed by independent methods (15, 19). This is fortunate because subcellular isolation methods are operationally simple and capable of broad application while other methods are not. This does not mean, however, that cell fractionation should be used to the exclusion of

[1] The survey of the literature pertaining to this review was completed in October, 1955.

[2] The following abbreviations are used in this chapter: ADP for adenosinediphosphate; AMP for adenosine-5-phosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; DNA for deoxyribonucleic acid; DPN for diphosphopyridine nucleotide; FAD for flavin-adenine-dinucleotide; RNA for ribonucleic acid; TPN for triphosphopyridine nucleotide; UDP for uridinediphosphate; UMP for uridine-5-phosphate; UTP for uridinetriphosphate.

other methods. Results obtained with any method must constantly be reassessed in the light of new developments, and, if possible, checked by independent methods.

The main finding of cell fractionation studies of peculiar interest to the biochemist has been the demonstration that a number of enzymes, coenzymes, and other compounds of biochemical interest are localized sharply in specific portions of the cell and not distributed haphazardly throughout (2, 10, 11). This has meant that prior to attempting purification of an enzyme, for example, isolating the subcellular component in which the enzyme was concentrated provided as great an advantage to the biochemist as had previously been afforded only by a judicious selection of tissues. Furthermore, isolation of subcellular structures has permitted the enzyme chemist to study the mechanism of enzyme reactions in systems uncomplicated by the extraneous influences of other cellular components. As a result it has become possible to study enzymes whose presence would probably have remained undetected in whole tissue extracts. The study of subcellular structures has consequently aroused great biochemical interest.

Methodology

General principles.—In previous papers (2, 11, 14, 20), we have formulated a number of criteria dealing with the isolation of cell components and the study of their biochemical properties. In our opinion, these criteria still hold for cell fractionation studies. One criterion, however, dealing with the establishment of the purity and cytological integrity of the isolated cell component, must now be modified in light of recent studies with the electron microscope. Findings with this instrument have shown, for example, that the mitochondria (21, 22), the secretory granules (23), the Golgi substance (23, 24), and the submicroscopic portions of the cytoplasm (22, 23, 25, 26, 27) all present characteristic and specific morphological aspects when examined in ultra thin sections of suitably fixed tissues. These observations thus provide new criteria for judging the purity and morphological integrity of isolated subcellular particulates. As we shall see, application of electron microscopic examination to isolated mitochondria and microsomes has already led to suggested improvements in centrifugation technique and demands that improved methods of isolation be devised in the future. It is too early, however, to foresee how profound an influence these new methods will have.

Isolation of nuclei.—A number of modifications of methods of isolating nuclei have been reviewed by Dounce (13) and need not be considered in detail here. At present the most suitable methods appear to be those using sucrose solutions supplemented with low concentrations of $CaCl_2$ or citric acid as the isolating media. One such method (28) permits the isolation of 70 to 90 per cent of the total nuclei of liver tissue with very low, and quantitatively measurable, contamination by intact cells and mitochondria. Such isolated nuclei also show excellent morphological preservation.

The revival of nonaqueous solvent methods for isolating nuclei (29, 30, 31), to prevent the loss of materials from the nucleus possibly occurring in aqueous media, has not met with wide application. In the first place, as pointed out elsewhere (11, 32, 33), the claim that water-soluble materials are lost from nuclei in aqueous media, has not been proved experimentally. In fact, evidence that one soluble enzyme is retained by nuclei during isolation has been obtained (32, 33, cf. 34).

Dounce and co-workers (35, 36) have recently observed that certain isolated nuclei form gels in dilute alkali and have proposed that the ability to form gels be used as a qualitative test of the suitability of nuclear isolation procedures. Although their observations are of considerable general interest, they have presented no experimental evidence on intact cells which would warrant the assumption that the formation of nuclear gels is a specific property of nuclei within the cell and an indicator of nuclear integrity. Their observation that an enzyme in mitochondria would prevent nuclear gelation does not prove that gelation is a characteristic property of the nucleus, since it is known that a soluble DNA depolymerase is localized in mitochondria (37) and since they observed that other treatments which would be expected to depolymerize DNA also weakened or abolished gelation. Furthermore, it should be noted that the nuclear isolation media disqualified by these workers on the basis of their gelation test need to be retested since cellular disintegration in these solutions was accomplished by methods known to cause mitochondrial rupture whereas in the acceptable media, cells were disrupted by more suitable procedures.

Isolation of mitochondria and microsomes.—Witter *et al.* (38) and Novikoff (39) have described methods for isolating mitochondria designed to preserve their morphological integrity as observed in ultra thin sections with the electron microscope. The former reported that 0.44 *M* sucrose adjusted to pH 6 with citric acid served this purpose while Novikoff used 0.25 *M* sucrose containing 7.5 per cent polyvinylpyrrolidone adjusted to pH 7.6. Novikoff also observed that his medium preserved the submicroscopic lamellar structures of the cytoplasm excellently and that the fluffy layer (cf. 2, 10) did not appear in his mitochondrial fractions. Palade & Siekevitz (40), on the other hand, have identified the microsomes as the submicroscopic cytoplasmic lamellae and isolated them from liver homogenized in 0.88 *M* sucrose. As will be mentioned later, however, investigators in the writers' laboratory have observed a number of other structures in the microsomal fraction (156).

Isolation of Golgi substance.—The Golgi substance was first observed in cells after their exposure to special fixation and osmication treatments. Although it was shown to have a characteristic morphology and to be widely distributed in different cell types, its history has been fraught with disagreement because of the inability to demonstrate its presence in unfixed cells. Recently, however, Dalton & Felix (24) were able to observe with the phase microscope, a structure within cells of the epididymis that had the characteristic morphology of the Golgi substance as seen in appropriately

fixed sections of this tissue. They showed (41) that this Golgi structure could be released intact from the epididymal cells, and subsequently Schneider & Kuff (42) isolated it from epididymal homogenates using a density gradient method. The isolated material was shown to have the same morphological and cytological properties as within the cell.

Biochemical studies of the isolated Golgi substance (42) have shown that it comprised only about 5 per cent of the total tissue nitrogen. It contained high concentrations of phospholipide which probably contributed to its low density and permitted its ready isolation. The isolated Golgi material also contained high concentrations of acid and alkaline phosphatase, and it dephosphorylated ATP much more rapidly than the other phosphorylated compounds tested. The material also contained high concentrations of RNA, a findings of some surprise since the Golgi substance of the epididymis did not show evidences of basophilia when examined cytologically. Cytochrome oxidase, DNA, deoxyribonuclease, and ascorbic acid were absent from the the isolated Golgi material.

Nucleolus and chromosomes.—Vincent (43) and Litt *et al.* (44) have reported the first methods developed for the isolation of nucleoli. The nucleoli were isolated by differential centrifugation from starfish oocytes and isolated liver cell nuclei, respectively, and have been studied mainly with respect to their nucleic acid content. The starfish nucleoli contained high concentrations of RNA and no DNA while the liver nucleoli contained both RNA and DNA, the latter in high concentration. Whether the DNA represented chromosomal contamination is a point that has not been finally settled. A recent report by Baltus (45) suggests that the nucleolus of the starfish oocyte may be the site of localization of nucleoside phosphorylase and the Kornberg DPN-synthesizing enzyme since these enzymes were 31 and 48 times as concentrated in the isolated nucleoli as in the oocytes. Unfortunately, enzyme assays were limited to the oocytes and the isolated nucleoli, and the validity of the assay methods was consequently not firmly established.

The question of the identity of the isolated "chromosomes" (46, 47) also remains unsettled. Although there is no doubt that structures resembling chromosomes cytologically have been demonstrated in such preparations, the fact remains that these structures cannot be seen either by ordinary, or by electron microscopy in the intact resting nuclei from which they were isolated. Until such an identification can be made or until chromosomes can be isolated from cells in which they are visible, the significance of the isolated chromatin material must remain undecided.

Mitotic apparatus.—Mazia & Dan (48) have described the isolation of the mitotic apparatus from dividing cells of sea urchin eggs. The procedure consists of "fixation" of the cells in 30 per cent ethanol at $-10°$C., separation of the mitotic apparatus from the cytoplasm by mechanical or chemical treatment, and isolation by centrifugation. Although the treatments used during isolation appear to be relatively mild, it remains to be established

whether they are, in fact, without effect. The isolated material has not been studied biochemically.

Secretory droplets.—The secretory granules occurring normally in tissue cells do not appear to have been isolated (cf. 2, 14). Droplets occurring in the cells of the kidney after injection of protein into an animal have recently been isolated in high purity by Strauss (49). These droplets possessed rather unique properties since they stained supravitally with both Janus Green B and neutral red. The droplets contained considerably lower concentrations of lipide phosphorus than kidney mitochondria and microsomes, somewhat lower concentrations of RNA, and they were especially distinguished by their high concentrations of acid phosphatase activity. The cytochrome oxidase and succinic dehydrogenase activities of the droplets were very low and this may be attributable to contaminating mitochondria. On the basis of the observed biochemical properties, it might be suggested that these droplets may be analogous to the hydrolytic mitochondria or lysosomes postulated by de Duve *et al.* (50) to be present in liver homogenates as a specific cellular element (see below).

The isolation of epinephrine-containing granules from the bovine adrenal medulla has been reported independently by Blaschko & Welch (51) and by Hillarp *et al.* (52). Although these granules appear to represent a highly specialized case inasmuch as they are the main particulate component of the bovine adrenal medulla, and classical mitochondria are absent from this tissue (53), their properties are of considerable interest. They stain with Janus Green as do normal mitochondria, but unlike mitochondria they do not stain with aniline acid fuchsin after fixation. The granules do, however, appear to contain all of the succinic dehydrogenase of the tissue (53). This property would again characterize them as mitochondria. In terms of quantity, the granules accounted for about one-third of the mass of the medullary cell and most of the epinephrine (adrenaline) and noradrenaline of the tissue was associated with them. (54) The concentration of sympathomimetic amines in the adrenal granules was very high: 4.3 per cent of the wet, and 19 per cent of the dry weight. The medullary granules, like the mitochondria of other tissues, also appeared to possess a semipermeable membrane, as shown by the fact that all of the amine content and a large part of the protein content of the granules was released in soluble form on exposure to hypotonic conditions (55).

The isolation of secretory granules from a glandular secretion (milk) has been reported by Morton (56). The isolated granules differed from the secretory granules visible in glandular cells under the microscope in that they were submicroscopic in size. The isolated milk "microsomes" were found to be lipoprotein complexes and contained the enzymes alkaline phosphatase, xanthine oxidase, diaphorase, and low concentrations of DPN-cytochrome-*c* reductase in addition to unidentified nucleic acid and hemochromogen components. Succinic dehydrogenase and cytochrome oxidase were absent.

Biochemical Composition of Subcellular Particulates

Much of the effort expended on the study of isolated cell components has been devoted to their enzymatic functions. It is possible here to consider certain recent developments in this field.

Nucleus.—The question as to whether succinic dehydrogenase and cytochrome oxidase are present in significant amounts in the nucleus has been under discussion since 1946 (57, 58, 65, 66) and now appears to have reached a final settlement with the confirmation by Dounce (13) that the presence of these enzymes in his isolated nuclear preparations is a result of contamination by mitochondrial fragments. Convincing evidence that this was the case had previously been presented by Hogeboom, Schneider & Striebich (28) who showed that the cytochrome oxidase content of highly purified nuclei was extremely low and was directly proportional to the number of mitochondria present.

Aside from these negative findings with respect to nuclear function, there has been the discovery that the enzyme that catalyzes the synthesis of DPN, according to the equation:

$$\text{Nicotinamide mononucleotide} + \text{ATP} \rightarrow \text{DPN} + \text{pyrophosphate}$$

is localized exclusively in the nucleus of the liver cell (32, 33). This is the first enzyme found in very high concentration in this structure and this suggests that the nucleus may be a center of coenzyme or nucleotide formation. The observation (59) that the pyrophosphorolysis of the uridine coenzymes, uridine diphosphoglucose (UDPG) and uridine diphosphoacetylglucosamine, by reactions analogous to the following

$$\text{UDPG} + \text{pyrophosphate} \rightarrow \text{UTP} + \text{Glucose-1-phosphate}$$

is localized in the rat liver nucleus suggests that the synthesis of these coenzymes may be localized in this cell structure as well. On the other hand, the synthesis of FAD from flavin mononucleotide and ATP by an analogous reaction does not appear to be a nuclear function at all since the enzyme responsible for this reaction has been recovered mainly in the soluble fraction of liver tissue, according to our own unpublished experiments.

One point that should be discussed with respect to nuclear function is the localization of the enzymes nucleoside phosphorylase and adenosine deaminase. Our own experiments (60), using fractionation in aqueous sucrose solutions, had indicated that these enzymes were present in the soluble fraction. On the other hand, Stern *et al.* (31), using organic solvents for the isolation of nuclei, have reported that these enzymes reach such high concentrations in the nuclei of some tissues as to suggest that they are localized exclusively in the nucleus. It is obviously difficult to reconcile the two findings. In the first place, Stern *et al.* did not observe these high enzyme concentrations in the nuclei of all tissues studied. They attributed this to differentiation of the nuclei in different tissues, an explanation that we prefer not to consider since it cannot be tested experimentally. The possibility that

the discrepant findings may be attributable to artifacts is, however, capable of experimental test. The possibility of inhibitors, for example, has not been ruled out in the experiments of Stern *et al.* since only the whole tissue and the isolated nuclei were tested for enzymatic activity. Furthermore, adsorption could account for the failure to observe a uniform enzyme distribution pattern in their experiments. On the other hand, the possibility exists that the enzymes had leaked from the nucleus during our own isolations. The experiments of Stern & Mirsky (34), which these authors claim to be direct evidence for leakage of enzymes from liver nuclei in sucrose solutions, in our opinion serve only to show that enzyme concentrations in nuclei isolated in aqueous solutions differ from those in nuclei isolated in organic solvents. They do not prove which experimental results are correct. On the other hand, the fact that the water-soluble DPN-synthesizing enzyme was localized exclusively in nuclei isolated in aqueous solutions, but was released when the nuclear membrance was disrupted or damaged (32, 33), makes it seem unlikely that enzyme leakage occurs in aqueous media.

With the exception of the synthesis of DPN, then, the function of the nucleus remains an enigma, the solution of which must await further research. As we shall see later, the nucleus may serve a further function in the synthesis of RNA. A possible role of the nucleus in control of oxidative phosphorylation reported by Johnson & Ackermann (61) now appears unfounded in view of recent experiments of Stern & Timonen (62). The latter observed that the ability of nuclear fractions to stimulate mitochondrial oxidative phosphorylation occurred only at low concentrations of mitochondria and could be duplicated by addition of heated nuclei or of albumin.

Krebs cycle.—As a result either of studies with isolated mitochondria or of mitochondria-containing preparations (e.g., cyclophorase), the concept has arisen that all of the enzymes of the tricarboxylic acid cycle are localized exclusively in mitochondria (cf. discussion, 11, 17). On the basis of data now available on the intracellular distribution of individual enzymes of the cycle, this concept is clearly untenable.

The earlier experiments (63, 64) on which this hypothesis rested were based upon measurements of oxygen uptake using various Krebs cycle substrates. Oxidation of these substrates was dependent upon the final participation of cytochrome oxidase and since this enzyme is localized exclusively in mitochondria (65, 66, 67), the oxygen uptake occurring upon addition of any of the Krebs cycle substrates would necessarily be confined to preparations containing mitochondria. As a result of this and related findings (20, 68, 69), it has become obvious that in order to determine the intracellular localization of an enzyme one must first of all develop a specific, direct method for determining the enzyme and then, in order to establish the validity of the enzymatic assay, carefully summate the amount of activity in all tissue fractions isolated.

Studies meeting the latter criterion lead to the following summary of the intracellular localization of Krebs cycle enzymes and coenzymes. Cyto-

chrome oxidase (65, 66, 67, 71), succinic dehydrogenase (65, 66, 67, 70, 71), fumarase (72), FAD (73), CoA (74), and cytochrome-*c* (75, 76, 77) are localized mainly or exclusively in isolated liver mitochondria. DPN and TPN (78), isocitric dehydrogenase (20), and aconitase (79) appear to be localized mainly in the soluble fraction of the tissue while the pyridine nucleotide-cytochrome-*c* reductases (20, 50, 71, 80) are associated with both mitochondria and microsomes. These findings suggest that a number of complex cytoplasmic interactions must occur for the cell to catalyze the tricarboxylic acid cycle reactions at a maximum rate. That such interactions do occur, despite the severe limitations one might expect to exist between particulate and soluble enzymes (resulting from spatial separation and the existence of membranes), has been demonstrated in numerous experiments *in vitro* in which cell fractions have been recombined in various permutations and combinations (20, 68, 69). These experiments have demonstrated that the cytoplasm as a whole is greatly superior to the mitochondria alone in catalyzing certain Krebs cycle oxidations.

It is apparent from the above summary that the localization of all of the enzymes of the Krebs cycle has not been studied. In this respect, Sanadi *et al.* (81) have reported the isolation from beef heart mitochondrial preparations of two of the enzymes involved in the oxidation of α-ketoglutarate. The isolation of other Krebs cycle enzymes from mitochondrial preparations has also been reported (82, 83). Although it would be attractive to conclude from these reports that these enzymes are also mitochondrial functions, such a conclusion would be completely unjustified unless confirmed by distribution studies on all fractions of the tissue. The studies of Siekevitz (68), in which oxidation of α-ketoglutarate by various liver fractions was measured, argue strongly against the exclusive localization of the enzymes reponsible for the oxidation of this substrate in mitochondria, since the rate of oxidation of the compound by isolated mitochondria proceeded only 20 per cent as rapidly as in the whole tissue.

There are two further points to be considered with respect to the diverse intracellular localization of the tricarboxylic acid cycle enzymes; one is the possibility that enzymes had leaked out of the mitochondria during their isolation and the other is that the cells may contain different types of the same enzyme (e.g., a soluble and a particulate isocitric dehydrogenase). The first possibility seems highly unlikely in view of the fact that a number of the enzymes found localized in mitochondria are soluble enzymes (37, 72, 84, 85, 86) that can be released as proteins of low molecular weight by disrupting the granules or subjecting them to hypotonic conditions. Furthermore, mitochondria are able to retain even smaller molecules than proteins during isolation (55, 87). The endogenous citrate present in the liver cell, for example, has been found to be localized in the isolated mitochondria and may be released by suspending the mitochondria in distilled water (87). Mitochondria also selectively retain certain anions and cations (88, 89). In view of this evidence, the possibility of leakage of enzymes during isolation seems remote.

With respect to the second possibility, the experiments of Dickman & Speyer (79) showed that the small amount of aconitase recovered in isolated liver mitochondria had pH optima at both 5.8 and 7.3 while the aconitase localized in the soluble fraction of the liver had greatest activity at 7.3. If the mitochondria were damaged, however, by freezing, their aconitase was released in soluble form and its pH optimum was at 7.3. Although these experiments suggest that there may be two types of aconitase in the liver cell, the alternative possibilities to be considered are that mitochondria may take up or release this enzyme during their isolation or that changes in pH have an effect on the permeability of the mitochondrial membrane to the substrate. Suitably designed washing experiments or experiments in which the soluble enzyme is added should permit a decision as to the correctness of these possibilities.

Recently de Duve *et al.* (50) have presented data suggesting that the mitochondrial DPN and TPN cytochrome-*c* reductases may be different from the pyridine nucleotide reductases associated with the microsomes. These authors observed that the mitochondrial reductases were partially inhibited by antimycin A whereas the microsomal enzymes were not. The mitochondrial enzymes were also dependent upon decreases in the tonicity of the medium for full activity while the microsomal enzymes were not. The latter observation is not surprising since it had previously been known that the activity of certain mitochondrial enzymes, such as glutamic dehydrogenase, was limited by the permeability of the mitochondrial membrane (84). The experiments with antimycin A are more difficult to interpret, however. Inhibition was never greater than 60 per cent and the antimycin A was apparently added at the same time as the diluent used to increase the permeability of the mitochondria. It would seem possible, therefore, that the effect might be a physical one rather than an actual inhibition of the enzymes. On the other hand, the observation of Strittmatter & Ball (71) that microsomes contained a specific hemochromogen, cytochrome-*m*, involved in the transfer of electrons from reduced DPN to cytochrome-*c* would support the contention of de Duve *et al.* if it could be demonstrated that mitochondria were entirely devoid of this pigment. Although Strittmatter & Ball state that cytochrome-*m* could not be detected in mitochondria, it seems possible that the presence of this pigment in their isolated mitochondria may have been obscured by the other cytochrome pigments present in these granules.

One final piece of experimental evidence bearing on this point needs to be considered. This is the isolation by Plaut & Sung (90) of a DPN-isocitric dehydrogenase from acetone powders of various tissue mitochondria. This finding supports the concept that different portions of the cell may contain different forms of the same enzyme. The activity of this enzyme is so low, however, that it cannot be detected in the presence of the TPN-isocitric dehydrogenase of whole tissue suspensions, and it leads one to question whether it is any more than a biochemical curiosity. For this observation to have real significance, it seems to us that the entire isocitric dehydrogenase

of mitochondria would have to be DPN-specific. Such does not appear to be the case.

Oxidative phosphorylation.—Studies of the mechanism of oxidative phosphorylation have aroused greater interest than any other type of study on cellular particles. Space does not permit the complete review that this subject warrants [cf. (91) for a recent review], and we shall mention only a few of the recent developments.

The direct demonstration by Lehninger and Lardy and their co-workers (92 to 95) that phosphate was esterified during the oxidation of reduced DPN and cytochrome-*c* was one of the most important findings of the past few years. According to their results, during the oxidation of reduced DPN, two molecules of inorganic phosphate were esterified per pair of electrons transferred to cytochrome-*c* while in the oxidation of reduced cytochrome-*c*, one molecule of phosphate was converted to high energy phosphate per pair of electrons transferred.

A number of compounds of biological importance other than AMP have also been found to be phosphorylated during mitochondrial oxidations. The phosphorylation of deoxynucleotides by respiring mitochondria has been reported by a number of workers (96, 97, 98). More recently Herbert *et al.* (99) in a study of UMP phosphorylation found that isolated liver mitochondria could phosphorylate UDP to UTP while the mitochondrial-free cytoplasm catalyzed the phosphorylation of UMP to UDP. The latter phosphorylations appear to involve transfer to phosphate from ATP rather than direct acceptance of phosphate during oxidation.

A new type of reaction occurring during oxidative phosphorylation was discovered by Cohn (100) in her experiments using inorganic phosphate labeled with O^{18}. She observed that the isotope was rapidly replaced by normal oxygen during the phosphorylation accompanying the oxidation of α-ketoglutarate, β-hydroxybutyrate, and succinate. The exchange of labeled oxygen closely followed the esterification of phosphate, but the number of phosphate molecules participating, assuming that a phosphate monoester was involved, was several times as great as the number of high energy phosphate bonds formed. The exchange reaction appeared to occur at each step of the electron transport system but not at the level of substrate oxidation. Boyer *et al.* (101) recently reported that washed rat liver mitochondria also catalyzed a rapid exchange of inorganic phosphate with ATP and an even more rapid exchange of the oxygens of inorganic phosphate with water in the absence of added substrates and of oxygen uptake. These authors postulated that oxygen was introduced into inorganic phosphate by a rapid reversal of the first step coupled to the electron transport system in which phosphate was esterified. This mechanism does not, however, satisfactorily explain some of the more recent results of Cohn & Drysdale (102) in which oxidative phosphorylation was studied using a medium in which either the inorganic phosphate or the water was labeled with O^{18}. It was observed that, although the rates of replacement of oxygen in the inorganic phosphate

were the same in either medium, the rates of replacing the oxygens in ATP were different in the two media. As a result of this finding the authors suggested that both oxygen and an unknown oxygen donor were involved in oxidative phosphorylation and that the mechanism of oxygen replacement might be different at the various points in the electron transport system where phosphate was esterified.

As we have pointed out previously, one of the great limitations in studies of isolated cell particulates such as mitochondria has been the inability to measure events as they occur within the mitochondrion because of a complete dependence upon determinations made on the surrounding medium. In a series of studies, Siekevitz & Potter (103, 104) have sought to define intramitochondrial events occurring during oxidative phosphorylation. In the earlier paper (103) they showed that the extremely low oxidative rate of freshly isolated mitochondria, in the presence of ATP and an oxidizable substrate, could be increased by an intramitochondrial event requiring ATP, namely, the synthesis of citrulline. This observation, together with previous findings in which it was found that the oxidative rate of mitochondria under similar conditions was controlled by the addition to the medium of either hexokinase and glucose or dinitrophenol (85, 105, 106, 107), indicated that mitochondrial oxidations were limited by the availability of phosphate acceptor. It also suggested that these oxidations were probably controlled within the cell by the supply of phosphate acceptor produced whether within or outside the mitochondria.

In more recent work (104), they have shown that isolated mitochondria contain constant amounts of a large number of acid-soluble compounds including nucleotides, coenzymes, and magnesium ions and that the loss of oxidative phosphorylation on incubation of mitochondria at 30°C. could be correlated with the loss of the compounds from the mitochondria. They conclude that these compounds are normally bound at specific sites within the mitochondria since they were unable to obtain reactivation of oxidative phosphorylation by addition of a high concentration of the material released from the mitochondria. Their reactivation experiment does not seem conclusive to us, however, since it ignores the possibility that undetected, essential factors were lost during the incubation (the citrate present in mitochondria is destroyed during such treatment, for example). Furthermore, if one assumes that some of the essential factors are localized in only a small area of the mitochondria, the concentrations added to obtain reactivation would have to be much higher than those used. Ernster & Löw (108) have in fact reported that the loss of phosphorylation accompanying succinate oxidation was attributable simply to an alteration of the calcium-magnesium ion ratio. They were able to restore phosphorylation partially by suitable adjustments in the concentrations of these ions. More recently Cooper *et al.* (109) obtained oxidative phosphorylation in a particulate fraction prepared from mitochondria by treatment with digitonin. These findings are of great interest since they permit the study of this reaction in the absence of the

physical barrier imposed by the mitochondrial membrane and offer hope that it will be possible to define more clearly the nature of oxidative phosphorylation reactions than has hitherto been possible.

In experiments using radioactive inorganic phosphate and involving isolation of intra- and extramitochondrial nucleotides, Siekevitz & Potter (104) observed that the turnover of ADP and ATP within the mitochondria was more rapid than their diffusion into and out of these granules. Furthermore they reached the conclusion that although mitochondria could absorb added ADP and AMP, they could not accumulate either of these nucleotides or retain the ATP they synthesized. The mitochondria appeared to act like secretory bodies for the production of ATP which could be used either at other sites within the mitochondria or outside the mitochondria for syntheses and other functions.

Fatty acid oxidation.—The initial observation (63, 110) that oxidation of fatty acids was confined to the mitochondria of the liver cell has been considerably extended from the biochemical point of view largely as the result of the finding that oxidation of these compounds could be reproduced in soluble extracts of mitochondria (111). As a result it has been possible to isolate and purify the enzymes involved in these oxidations and to show that five steps were involved (112, 113, 114). These included activation of the fatty acid (by formation of a CoA derivative), oxidation, hydration, reoxidation, and cleavage to acetyl CoA and a fatty acid shortened by two carbon atoms. Each of the enzymes involved has been isolated from mitochondrial preparations (112). It would be attractive to conclude from these isolation experiments and from the earlier measurements of cell fractions (63, 110) that the fatty acid oxidation enzymes are localized exclusively in mitochondria. Since the assay involved oxygen uptake determinations, it is apparent that these results should be re-examined from the cytochemical standpoint, especially since the individual enzymes involved are now known and can be measured separately. The steps in the oxidation of fatty acids should also be examined from the standpoint of their localization within the mitochondrion, since it may well be that the various enzymes involved are spatially separated from each other.

Fatty acid formation.—Biosynthesis of fatty acids has been postulated to occur by reversal of the fatty acid oxidation sequence (113). *In vitro* measurements of the incorporation of labeled acetate into liver fatty acids offer some support for this hypothesis since mitochondria appear to be required for incorporation to occur (115). However, a particle-free, soluble tissue fraction is also needed. Fatty acid biosynthesis has also been shown to require ATP, CoA, and DPN in suitably treated soluble extracts of acetone powders of mitochondria (116). This would, of course, also agree with the proposed mechanism.

In the mammary gland, however, fatty acid synthesis would appear to be independent of mitochondria since incorporation of acetate into the fatty acids of this tissue occurred in the particulate-free soluble fraction (117).

Cholesterol metabolism.—The formation of cholesterol as measured *in vitro* by incorporation of labeled acetate into tissue cholesterol also involves two tissue fractions. Again the soluble fraction is required, but in this case the large microsomes appear to contain the additional enzymes or coenzymes required (118, 119, 120).

It is of interest to mention in connection with these studies on cholesterol biosynthesis, that free cholesterol is found localized mainly in the microsomes of liver tissue while esterified cholesterol has been recovered mainly in the fatty fraction that rises in the top of the centrifuge tube when liver homogenates are centrifuged at high speed (121). Cholesterol esterase, on the other hand, is localized in the microsomes (122). Vitamin A ester and its esterase are reported to be similarly separated in their intracellular locale (123, 124).

Lecithin formation.—The formation of lecithin appears to take place in the liver by two independent methods involving collaboration of the mitochondria and some other cytoplasmic factor (125 to 128). In one, choline is directly incorporated into the liver lecithin while in the other, phosphorylcholine is transformed into lecithin, apparently after interaction with cytidine triphosphate. Both incorporation reactions require concurrent oxidative phosphorylation.

Steroid metabolism.—Hydroxylation of deoxycorticosterone at the 11 carbon atom has been shown to occur *in vitro* in adrenal mitochondrial preparations and to be maintained by oxidative phosphorylation (129, 130, 131). More recently, the enzyme has been obtained in more purified preparations from extracts of acetone powders (132). In such extracts oxidative phosphorylation is unnecessary, and the only cofactors needed are fumarate, TPN, and oxygen.

The oxidation of progesterone, on the other hand, has been reported to be catalyzed by the soluble fraction of the adrenal (133). Both ATP and DPN are required, and oxidation proceeds via two pathways in one of which deoxycorticosterone is the product, while in the other 17-hydroxy-11-deoxycorticosterone is produced, with the intermediate formation of 17-hydroxyprogesterone.

Intermediate between these results with adrenal fractions, are those of Recknagel & Glenn who reported that an enzyme in rate liver capable of degrading the 17,21-dihydroxy-20-ketone side chain of adrenocortical steroids was equally distributed between the microsomes and the soluble fraction (134).

Ribonucleic acid.—The distribution of RNA in the cell has been reviewed elsewhere and need not be considered in detail here (2, 11, 14). There appears to be general agreement that the greatest amount of RNA is localized in the microsomes of liver tissue and that both the turnover rate and the nucleotide composition of nuclear RNA are markedly different from those of cytoplasmic RNA. The earlier data (135), however, which indicated that RNA was synthesized in the nucleus and then transferred to the soluble

cytoplasm where it was built first into the microsomes and then into the mitochondria has not received confirmation from more recent work in which the rate of incorporation of various compounds into these cellular components has been studied (136, 137). The question as to whether the nucleotide composition of the mitochondrial, microsomal, and soluble RNA differ is also in dispute, although the bulk of the evidence on liver tissue of the same species suggests that there is no striking difference (138 to 141).

Some recent *in vitro* studies on the enzymatic formation of RNA precursors indicate that at least some of these steps are not localized in the nucleus. According to Reichard (142) the sequence of reactions leading to the formation of orotic acid:

$$NH_3 + CO_2 + \text{aspartate} \xrightarrow[\text{ATP}]{\text{glutamate}} \text{ureidosuccinate} \rightarrow \text{orotic acid}$$

appears to take place in the mitochondria of rat liver. On the other hand, Hurlbert & Reichard (143) have reported that the conversion of orotic acid to uridine phosphates, which are intermediates in the *in vivo* synthesis of RNA, is localized in the soluble fraction of liver tissue.

Protein formation.—The synthesis of protein as measured *in vitro* by incorporation of labeled amino acids into liver protein had been extended somewhat by recent work. In earlier investigations (69) it was shown that incorporation occurred most extensively in the microsome fraction but that simultaneous oxidative phosphorylation by mitochondria was also necessary. More recently, Zamecnik & Keller (144) eliminated the mitochondrial requirement by adding small amounts of the soluble liver fraction and cofactors permitting glycolysis. These workers also found that the reaction was strongly inhibited by ribonuclease. It can be concluded that incorporation of amino acids into proteins is an enzymatic function of the microsomes but that this function is controlled by the availability of energy produced in other parts of the cell.

Detoxifications.—Recent experiments on the metabolism of various drugs has implicated the microsomes as a major site of detoxification. The earlier work of Mueller & Miller (145) showed that demethylation of certain azo dye carcinogens was catalyzed by the microsomes and soluble fraction of the liver in the presence of TPN and DPN. La Du *et al.* (146) have shown that dealkylation of a large number of compounds, e.g., aminopyrine, is catalyzed by the microsomes in the presence of TPN and oxygen and the soluble fraction. The function of the soluble fraction is apparently to keep the TPN reduced, since it can be replaced by the addition of glucose-6-phosphate dehydrogenase. The requirement for oxygen is not understood. However, deamination of drugs such as amphetamine (147) and oxidation of barbiturates (148) is also catalyzed by isolated microsomes and has identical cofactor requirements.

Comments on enzyme distributions.—It is apparent from the above summary of recent work on the enzymatic properties of subcellular particulates

that many of the studies leave much to be desired from the cytochemical standpoint. In the first place, a large proportion of the reactions studied have merely been detected in certain cellular fractions. Such qualitative findings may give an entirely different picture from that eventually obtained when the enzyme or enzymes involved can be measured quantitatively and determined in all cell fractions.

Furthermore, many of the reactions studied really consist of an unknown number of component steps. Until the mechanism of such reactions can be clarified and the individual steps measured in cell fractions, final judgment on the significance of the intracellular localization of the over-all reaction must be reserved, since we have already seen the dangers that can arise from such determinations in the enzymes of the Krebs cycle.

In the case of the reactions measured by incorporation of isotopically labeled compounds there is an additional complication since the reaction product is a component of the tissue itself and may determine the site of the reaction. We may, for example, inquire whether the localization of the cholesterol-forming enzyme in microsomes is attributable to the fact that the majority of the liver cholesterol is localized in these particles. It will not be possible to decide this point until this and the other reactions mentioned above which depend upon incorporation measurements can be studied under circumstances permitting actual net synthesis. The experiments of Ullmann & Straub (149) on synthesis of enzymes in cell-free tissue homogenates are an encouraging step in this direction.

These comments should not be construed to imply that measurements of the types discussed are of no value. On the contrary, such studies have been extremely useful in providing the first means by which the metabolism of these important cellular compounds could be investigated. The difficulties in reaching precise cytochemical conclusions from such studies would seem apparent, however.

Biochemical heterogeneity of subcellular particles. Cytological examination of isolated cellular structures such as mitochondria indicated that all the particles in an isolated mitochondrial preparation exhibited similar staining and optical properties even though they were highly pleomorphic (70). The possibility nevertheless existed that such isolated fractions could consist of biochemically distinct collections of particles. A number of experiments have been performed which indicate that this is actually the case.

In the experiments of Novikoff *et al.* (150), liver homogenates were centrifuged at gradually increasing speeds, and the sediments were examined cytologically and biochemically. The results showed that certain enzymes such as uricase appeared to be associated with smaller granules than the mitochondria in which they had previously been localized (60, 151). This was confirmed by Thomson & Mikuta (152), using an entirely different method. These workers correlated enzymatic activity with particle size as determined by centrifugation in a sucrose density gradient. They found that uricase appeared to be associated with a particle distinct from those in which

succinic dehydrogenase, cytochrome oxidase, and DPN-cytochrome reductase were localized. These findings were extended in the experiments of Paigen (153), Kuff & Schneider (10, 154), and Holter *et al.* (155) in which isolated mitochondria were further fractionated by differential centrifugation, or by packing in high centrifugal fields, or centrifugation in a density gradient. Kuff & Schneider isolated granules which contained most of the uricase and deoxyribonuclease activities of the mitochondrial fraction and in which the concentration of these enzymes was 15 to 20 times as great as in the original tissue. More recently de Duve *et al.* (50) have suggested that these granules be called lysosomes because they contained a number of hydrolytic enzymes such as acid phosphatase, ribo- and deoxyribonuclease, cathepsin, and β-glucuronidase in high concentrations in addition to uricase. These workers have also suggested a modification in the usual cell fractionation procedure by means of which these granules can be concentrated in a fraction intermediate between the mitochondria and microsomes.

Although it seems clear that some of the enzymes previously assigned to mitochondria are actually localized on different granules, considerable work remains to be done before the significance of this result can be assessed. The granules need to be isolated and identified cytologically. Based on what we know now, these granules could be small mitochondria. Recent experiments of Kuff *et al.* (156) in which electron microscopic observations were correlated with sedimentation rates of various liver enzymes suggested, however, that uricase and acid phosphatase may be associated with certain of the vesicles derived from the submicroscopic lamellar structures located in the cytoplasm. Other "microsomal" structures, not at present identifiable cytologically, were also observed.

One final point that should be considered is the nature of the "fluffy" layer that is regularly observed to sediment above the mitochondrial pellet (2). Although the bulk of the available evidence favors the view that this material consists mainly of submicroscopic particles (cf. 2, 10 14), it is readily understandable that some workers have reached the conclusion that this layer is a biochemically distinct subcellular fraction (157) or that it contains biochemically distinct small mitochondria (158). Microscopic examination of this fraction readily reveals that the fluffy material contains both mitochondria and large numbers of submicroscopic particles (2). It is clear therefore that this fraction could appear biochemically distinct from either mitochondria or microsomes and that biochemically distinct small mitochondria could be isolated from this material. This does not constitute sufficient evidence, in our opinion, for considering the fluffy layer to be a specific subcellular element. The fraction is too obviously cytologically heterogeneous for such a conclusion. As a matter of fact, the recent preliminary report of Novikoff (39), that liver homogenates prepared in sucrose-polyvinylpyrrolidone media are devoid of fluffy material, may eventually lead to the conclusion that the fluffy layer is an artifact.

Submicroscopic Organization of Subcellular Particulates

Recent observations with the electron microscope that mitochondria have a characteristic organization consisting of a surface membrane and internal compartments separated by lamellae (21, 22), are important for other reasons than as a means of identification.

In the first place, the demonstration of a membrane offers direct support for certain biochemical observations best explained on the assumption that the mitochondrion is surrounded by a semipermeable barrier. A number of mitochondrial enzymes, such as acid phosphatase (86), adenosinetriphosphatase (85), glutamic dehydrogenase (84), DPN-cytochrome-*c* reductase (50, 92), ribonuclease (50, 60), and deoxyribonuclease (50, 60), exhibit a latent behavior when the mitochondria are isolated under the most favorable conditions and the enzyme activities are measured under conditions of tonicity similar to those used during isolation. Full activity of these enzymes is reached only when the mitochondria are either completely disrupted or damaged by treatments such as freezing or unfavorable osmotic conditions. The best explanation of these results is that the mitochondria, in the freshly isolated condition, are impermeable to the substrates of these enzymes.

Several other lines of evidence point to the presence of a semipermeable mitochondrial membrane. The fact that soluble proteins (86, 159, 160) and small molecules such as citrate (87), nucleotides, and coenzymes (55, 104) are retained by mitochondria during their isolation but are readily released when the mitochondria are exposed to hypotonic solutions or temperatures of 30°C. is suggestive of such a membrane, since both of these treatments cause mitochondrial swelling. Moreover, the fact that mitochondria are able to concentrate hydrogen, sodium, magnesium, and phosphate ions (88, 89) while undergoing active metabolism *in vitro* is strong evidence for this view. Finally, Tedeschi & Harris (161) studied the permeability of isolated mitochondria to nonelectrolytes and showed that these granules obey quantitatively the osmotic law of Boyle and van't Hoff and that the rate of penetration of the compounds was correlated with their distribution between olive oil and water. These authors conclude that mitochondria have a semipermeable lipide membrane.

Before concluding the discussion of the mitochondrial membrane, it is necessary to consider once again the views of Harman (162, 163) who believes that mitochondria are gels and do not possess a surface membrane. His evidence for this view is based on the following: (*a*) pigeon skeletal muscle mitochondria do not lyse when suspended in water, (*b*) these mitochondria do not release significant amounts of nitrogen when suspended in water, and (*c*) mitochondria with damaged surfaces will show osmotic activity. With respect to the first point, mitochondrial lysis has been observed by a number of workers (cited in 10), although these mitochondria were obtained from a different tissue than that used by Harman. With regard to the loss of nitrogen, our own unpublished experiments have shown that about 50 per cent of the total nitrogen of liver mitochondria is released when

they are suspended in water and resedimented. Furthermore, similar observations have been reported with the isolated bovine adrenal granules (55). The results of Dianzani (164) quoted by Harman in support of his findings showing no loss of nitrogen, do not appear to have dealt with release of nitrogen from mitochondria but did show the loss of a considerable amount of nucleic acid from liver mitochondria in water. Dianzani concluded that the presence of a semipermeable mitochondrial membrane was highly probable. The evidence presented by Harman for the third point seems rather weak and controversial to us. In view of the large amount of evidence favoring the presence of a semipermeable membrane in the mitochondria of tissues such as liver, kidney, adrenal, and insect muscle (*vide supra* and 165), the opposing findings of Harman may represent a special case.

Mitochondria are composed of almost equal amounts of soluble and insoluble particulate material (55, 159, 160) which is a finding of great interest in relation to the electron microscopic observations. Taken together with the fact that certain mitochondrial enzymes are soluble while others are strongly bound to the particulate material, the results imply a highly organized arrangment of enzymes within the mitochondrion. The results obtained so far, for example, indicate that a number of enzymes are firmly bound to the insoluble structural matrix of the mitochondrion. These include adenosinetriphosphatase, succinic dehydrogenase, cytochrome oxidase, DPN-cytochrome-*c* reductase, uricase, and cytochrome-*c* (60, 159, 166). With the exception of the last-mentioned, the findings are perhaps not surprising to the biochemist since he has long been familiar with these particular enzymes as insoluble ones. The fact that cytochrome-*c*, a soluble protein of low molecular weight, should be associated with the structural components of the mitochondria is surprising, however, except when considered from the standpoint that it is localized in the same mitochondrial components as some of the enzymes with which it reacts.

On the other hand, a number of other mitochondrial enzymes that also would be expected to interact with the structurally bound ones, appear to exist in the mitochondrion in a soluble or diffusible form. Glutamic dehydrogenase (84), adenylate kinase (85), and fumarase (72) are examples. Siekevitz & Potter (104) have, in fact, concluded from their studies that there may be two adenylate kinases in the mitochondrion, one at the surface and another in the interior at the site of oxidative phosphorylation.

From these biochemical results and from the morphological findings with the electron microscope, the mitochondrion would seem to be a highly organized structure in which the various enzymes are so localized that the products of one reaction are formed at an appropriate site for the next enzymatic transformation. Furthermore, because of the exceedingly great diversity of mitochondrial functions and the fact that more than one mitochondrial enzyme may require the same substrate or cofactor, it seems likely that certain of the enzymes of the mitochondrion that deal with more generalized biochemical functions are distributed throughout the mitochondrion

either diffusely or at a number of points. Other enzymes dealing with highly specialized reactions, may be localized in only a very small area of the mitochondrion.

It is also evident that the "microsome" problem is unsettled and requires, for definition, the skills of both the biochemist and the morphologist, at present particularly that of the latter.

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CHEMISTRY OF THE FUNGI[1,2]

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An article by Birkinshaw (1) on the chemistry of fungi was included in the *Annual Review of Biochemistry* for 1953. Since that date the field has not been covered from the same point of view, although much relevant information is contained under the heading of "Biochemistry of Antibiotics" in last year's *Review* [Binkley (2)]. The vigorous search for antibiotics produced by microorganisms continues to produce much unsorted information concerning the metabolism and metabolic products of fungi and actinomycetes. It is giving, however, a somewhat lop-sided picture of the general field of metabolism, especially in the case of the actinomycetes, since organisms which do not produce any biologically active material are not investigated further. Even when antibiotics are discovered, usually little attention is paid to the accompanying biologically inactive material, much of which is undoubtedly of chemical and biochemical interest.

It is the aim of the reviewers to report and collate the work published since Birkinshaw's review, including earlier work, if it is especially relevant. The attempt has been made to bring some order into what would otherwise be a mere catalogue of names and structures, and to discuss the biogenesis of the fungal metabolic products. The actinomycetes are normally considered to come under the heading of bacteria, and no attempt will be made to consider their chemistry in full. However, where the metabolic products of actinomycetes can be related to those of fungi, they are included in this article.

The grouping of fungal products is difficult. While some compounds, such as the anthraquinones, fall naturally into a coherent group, others seem to defy rational arrangement; the range and diversity of fungal metabolic products is almost as wide as that of organic chemistry itself. It is the intention of the authors to bring out the structural relationships in this varied field, and, to that end, the classification which follows is based on the main structural features of the compounds in question and is not a strict chemical classification such as that used in "Beilstein." Thus, for instance, the presence of a phenyl group will not necessarily prevent inclusion of a compound with other aliphatic substances, if its main features are aliphatic. Nevertheless, a fair amount of cross-reference from one group to another is necessary to our purpose and, in fact, serves to emphasize the interrelationships within the whole field.

[1] The survey of the literature pertaining to this review was concluded in September, 1955.

[2] A list of species cited in the text with the authorities for each is given at the end of the text.

Each section carries an introductory paragraph setting out the advances made under this heading, followed by detailed treatment of the individual substances under side-headings. The choice of heading has been, of course, governed by the new work available for review. Other groups of fungal products exist; several are described by Raistrick (3). No claim is made to have covered the whole of the progress in this field. The authors have chosen those areas in which, in their view, the most significant developments have taken place.

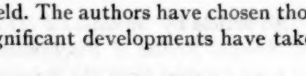
Some aspects of the subject are considered in recent books by Robinson (4) and Bracken (5).

POLYENES

Many products from a wide range of fungi contain a more or less extended conjugated double-bond system. Two simple polyene acids have been isolated recently from higher fungi, namely corticrocin (I) and cortisalin (II), containing respectively 6 and 7 double bonds in conjugation. Limocrocin (III), an actinomycete pigment, has been shown to be very probably a substituted amide of tetradecaheptaene-1,14-dicarboxylic acid, the next longer member of the series to corticrocin. Fumagillin, from *Aspergillus fumigatus*,[2] is apparently a mono-ester of octatetraene-1,8-dicarboxylic acid (6). Some other fungal products contain short polyene side-chains; sorbicillin, muscarufin (IV), and thelephoric acid (7) have a diene side-chain, and auroglaucin (see p. 234) almost certainly has a triene side-chain.

$HOOC(CH:CH)_6 \cdot COOH$

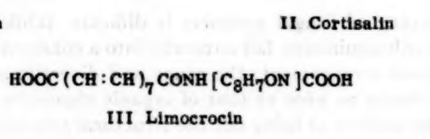
I Corticrocin



II Cortisalin

$HOOC(CH:CH)_7CONH[C_8H_7ON]COOH$

III Limocrocin



IV Muscarufin

Erdtman (8) points out that muscarufin (9), the red pigment from *Amanita muscaria*, accommodates the whole molecule of corticrocin and that

thelephoric acid from *Thelephora palmata* (closely related to *Corticium*) carries the same diene-oic side-chain.

Carotenoids, which are produced by several fungi, will not be considered here. A review of some biochemical aspects of fungal carotenoids by Haxo has appeared very recently (10), and the subject was also covered in the *Annual Review of Biochemistry* in 1955 by Goodwin (11).

Corticrocin.—Erdtman (8, 12) isolated from a mycorrhizal mycelium growing on the roots of various conifers, etc., and probably belonging to the fungus *Corticium croceum*, a yellow pigment, corticrocin, which he identified as I, probably the all-*trans*-isomer. Shaw & Whiting (13, 14) and Weedon (15, 16) have now confirmed this constitution by synthesis.

Cortisalin.—Gripenberg (17) isolated a red-violet pigment from *Corticium salicinum*, and identified it as II mainly on the basis of its hydrogenation to 14-*p*-hydroxyphenyltetradecane-1-carboxylic acid, identical with a synthetic specimen (18). Cortisalin itself has now been synthesised by Marshall & Whiting (19), thus confirming the structure II.

Limocrocin.—Brockmann and co-workers (20, 21) have shown that *Streptomyces limosus* produces, on a glycine-glycerol medium, a yellow pigment (a polyene, probably $C_{25}H_{24}O_6N_2$) which gives a deep blue colour in sulphuric acid, and absorbs seven molecules of bromine. It is readily hydrogenated to form colourless perhydrolimocrocin, $C_{25}H_{38}O_6N_2$, which no longer gives the blue sulphuric colour. Limocrocin and perhydrolimocrocin contain two carboxyl groups and show no basic properties. A band in the infrared spectrum of perhydrolimocrocin is consistent with the presence of an amide group, and alkaline or acid hydrolysis yields tetradecane-1,14-dicarboxylic acid. The most probable formula for limocrocin is therefore III, where the C_8H_7ON residue is probably a bicyclic heterocyclic system.

Sorbicillin.—The structure of this product of *Penicillium notatum* has been confirmed by synthesis by Kuhn & Staab (22).

Acetylenic Compounds

Further work is reported on the structure of several polyacetylenic compounds, a group of substances which has been recognized for some time in cultures of higher fungi. A fungal compound with a single acetylene link attached to an aromatic heterocyclic system has also been isolated and identified. A possible biogenetic connection between the new compound, junipal, and the polyacetylenes is mentioned below.

All the products in this group have been obtained from higher fungi except mycomycin, a product of an actinomycete. No acetylenes are known from lower fungi.

Polyacetylenic compounds.—Bu'Lock (23) has investigated a large number of higher fungi in a search for polyacetylenic compounds, detected by their distinctive ultraviolet absorption spectra. Anchel (24) has carried out a search for antibiotics in basidiomycete culture filtrates, and certain of these are similarly associated with the characteristic ultraviolet absorption spectra of polyacetylenes.

Bu'Lock *et al.* (25) assign to nemotin and nemotinic acid, produced by *Poria tenuis*, *P. corticola*, and an unidentified basidiomycete, B. 841, the structures V and VI in Table I, closely similar to that of mycomycin, VII (26), a product of the actinomycete *Nocardia acidophilus* and containing the unusual allene grouping. It should be noted that nemotin and nemotinic acid readily polymerise in the solid state. Hence all work has of necessity been done in solution.

TABLE I
FUNGAL POLYACETYLENES

V	Nemotin	$HC{\equiv}C{-}C{\equiv}C{-}CH{=}C{=}CH{-}CH{-}CH_2{-}CH_2{-}CO$
VI	Nemotinic acid	$HC{\equiv}C{-}C{\equiv}C{-}CH{=}C{=}CH{-}CHOH{-}CH_2{-}CH_2{-}COOH$
VII	Mycomycin	$HC{\equiv}C{-}C{\equiv}C{-}CH{=}C{=}CH{-}CH{=}CH{-}CH{=}CH{-}CH_2{-}COOH$
VIII	Biformyne I	$HC{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}CH_2{-}CHOH{-}CH_2OH$
IX	Matricarianol	$H_3C{-}CH{=}CH{-}C{\equiv}C{-}C{\equiv}C{-}CH{=}CH{-}CH_2OH$
X		$HOOC{-}CH{=}CH{-}C{\equiv}C{-}C{\equiv}C{-}CH{=}CH{-}COOH$
XI	Matricaria ester	$H_3COOC{-}CH{=}CH{-}C{\equiv}C{-}C{\equiv}C{-}CH{=}CH{-}CH_3$
XII	Diatretyne I	$H_2NOC{-}C{\equiv}C{-}C{\equiv}C{-}CH{=}CH{-}COOH$
XIII	Diatretyne II	$N{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}CH{=}CH{-}COOH$
XIV	Agrocybin	$H_2NOC{-}C{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}CH_2OH$

Polyporus anthracophilus produces a series of C_{10} polyacetylenes [Bu'Lock, Jones & Turner (27)]. From the culture filtrate have been isolated *trans-trans*-matricarianol (IX), an ene-diyne-ene first isolated from Compositae by Holme & Sörensen (28), and also the dicarboxylic acid X. From the mycelium there was obtained the *trans-trans*-matricaria ester (XI); this is not known as a plant product, but the *cis-cis-* and 2-*trans*-8-*cis*-isomers are known Compositae products. Bu'Lock *et al.* (27) suggest that these close similarities between plant and fungal products indicate that essentially similar dehydrogenation mechanisms are at work.

The structures of the products diatretyne I (XII) and diatretyne II (XIII) from *Clitocybe diatreta* have been clarified (29). The antibiotic activity previously reported for diatretyne I is now shown to be attributable to contamination with the active nitrile diatretyne II. This is believed to be the first natural polyacetylenic nitrile reported. Bu'Lock *et al.* (30) have synthesized diatretyne I and also agrocybin (XIV), an antibiotic from *Agrocybe dura*. Bu'Lock & Jones had formerly (31) adduced evidence for a terminal CH_3 group in place of the CH_2OH, but synthesis of this CH_3 compound showed that it was not identical with agrocybin.

The nomenclature of the antibiotics from ***Polyporus biformis*** is confusing. Originally "biformin" and "biforminic acid" were reported by Robbins *et al.* (32). Reinvestigation by Anchel & Cohen (33) of this fungus resulted in the isolation of a polyacetylene, for which they retained the name "biformin" [this is referred to in last year's review (2) as "biformin 1"]. Biformin is now altered to "biformyne I" (24), and a second substance "biformyne II" is reported from the same culture but freed from it only on boiling. This latter substance is stated to be a C_{10} compound. According to a review article by Bohlmann (34), biformyne I probably has the formula designated as VIII.

The fungus *Drosophila subatrata* produces four antibiotic substances (35), two of which, drosophilin C and drosophilin D, are probably polyacetylenes. These are said to undergo changes in alkali similar to those observed for nemotin and mycomycin (36).

The formulae of those fungal polyacetylenes which are established are shown in Table I. It seems probable that the members of this series of polyacetylenes are closely linked biogenetically, and it is especially interesting that the actinomycete product, mycomycin, should fit so well into a group mainly derived from higher fungi and plants.

Junipal.—Birkinshaw & Chaplen (37) have isolated, from the wood-rotting fungus *Daedalea juniperina* grown on a glucose-Marmite medium, what is believed to be the first example of a thiophene derivative among fungal metabolic products. The compound contains C-methyl and aldehyde groups. It can be oxidised in two stages to the known thiophene-2,5-dicarboxylic acid. The constitution XV is confirmed by the ultraviolet and infrared absorption spectra.

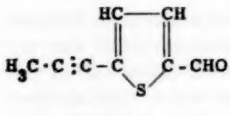
Anisaldehyde, isolated earlier from two other species of wood-rotting fungi, is also produced along with junipal by *D. juniperina.* The authors suggest tentatively that the two compounds may be formed from a common straight-chain precursor, a C_7 unsaturated aldehyde. This would also provide a possible biosynthetic relation with the other straight-chain acetylenic compounds mentioned above.

Alicyclic Compounds

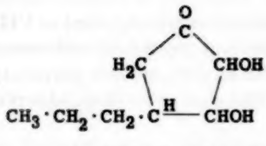
The structure of terrein has been established, and its stereochemistry elucidated. The relationship between gibberellin A and gibberellic acid is described, and progress towards the structure of gibberellic acid is reported. The structure of caldariomycin is confirmed.

The triterpenes occurring in higher fungi form a closely related group. They will not be described here, since they have been very recently reviewed in detail by Jones & Halsall (38).

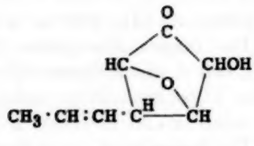
Terrein.—The optically active α-ketol, terrein, was isolated by Raistrick & Smith (39) from two strains of *Aspergillus terreus.* It was subsequently shown (40) that tetrahydroterrein has the structure XVI and that terrein itself has a propenyl side-chain. Of the various possible formulae for terrein, the two selected as most probable were XVII and XVIII. Of these, XVII was



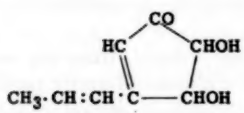
XV Junipal



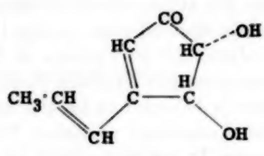
XVI Tetrahydroterrein



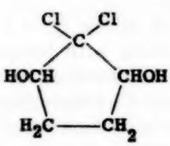
XVII



XVIII



XIX Terrein



XX Caldariomycin

preferred because: (*a*) optically active *n*-propylsuccinic acid is obtained on oxidation of tetrahydroterrein; (*b*) tetrahydroterrein loses water readily to give a diketone whereas terrein does not; (*c*) terrein gives only a mono-*p*-bromobenzoate, and (*d*) terrein does not react with maleic anhydride.

Barton & Miller (41) have now shown that XIX is nevertheless the correct formula. Terrein forms a mono-dinitrophenylhydrazone which gives a di-O-acetate, and all three compounds have ultraviolet and infrared absorption spectra in agreement with formula XIX. This conclusion is confirmed on spectral grounds by Grove (42), who also obtained rather inconclusive evidence of the reaction of terrein with maleic anhydride.

The stereochemistry is shown to be as in XIX by Barton & Miller, who converted terrein to a derivative of (+)-tartaric acid. The *trans* configuration of the exocyclic double bond is shown by infrared absorption spectra.

Gibberellin A and gibberellic acid.—Gibberella fujikuroi, the causative organism of Bakanae disease in rice, was shown by Yabuta and co-workers (43 to 47) to synthesize a compound which produces the elongation of the shoots of rice seedlings characteristic of the disease. This metabolite, m.p. 242–44°C. was first named gibberellin B, but now gibberellin A. The formula proposed was $C_{22}H_{26}O_7$.

Curtis & Cross (48), working with the same species grown on Raulin-Thom sucrose medium, failed to obtain gibberellin A but have isolated a different but closely related substance with similar growth-promoting properties [see Brian *et al.* (49, 50)] which they have named gibberellic acid. It melts at 233–35°C. and is well-characterised as $C_{19}H_{22}O_6$. Stodola *et al.* (51) have isolated a mixture of gibberellin A and gibberellic acid (the latter they call gibberellin X) from cultures of the same organism.

Gibberellic acid appears to contain carboxyl, lactone, hydroxyl (primary or secondary) and tertiary-hydroxyl groupings, and probably two double bonds, but no aromatic ring. This suggests four alicyclic rings [Cross (52)]. Gibberellic acid and gibberellin A have several degradation products in common. Thus on mild acid hydrolysis gibberellic acid yields CO_2 and an unsaturated tetracyclic hydroxyacid, allogibberic acid, identical with gibberellin B, which is similarly obtained from gibberellin A. More vigorous acid hydrolysis yields gibberic acid, a tetracyclic ketoacid, also obtained by acid-catalysed rearrangement of allogibberic acid. Both allogibberic and gibberic acids have the formula $C_{18}H_{20}O_3$ [not $C_{19}H_{22}O_3$ as reported by Yabuta *et al.* (45, 46)] and contain one benzene ring. The two acids and the parent gibberellic acid all yield, on dehydrogenation, gibberene, $C_{15}H_{12}$, shown by synthesis to be 1,7-dimethylfluorene [Mulholland & Ward (53)].

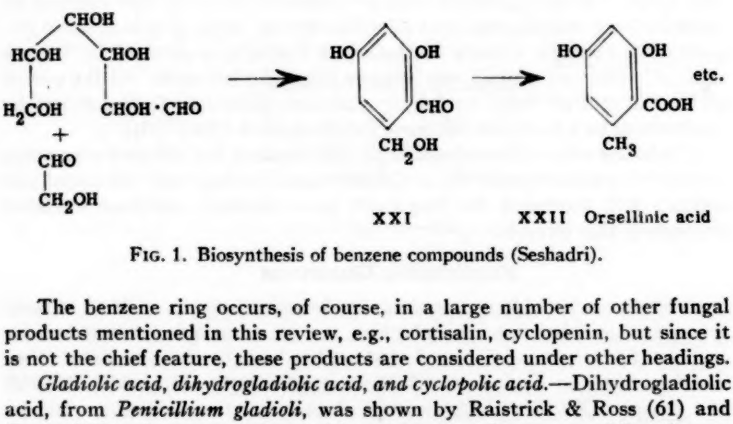
Caldariomycin.—Clutterbuck *et al.* (54) isolated the chlorine-containing metabolite caldariomycin from *Caldariomyces fumago* and advanced the formula XX. Barton & De Mayo (55) have obtained additional evidence supporting this formula.

Benzene Compounds

A large number of fungal products are benzene derivatives. Most of these are substituted phenols or phenol ethers, and many are closely related to one another. The structure of several of these has been further elucidated. New members of the group include 2,5-dihydroxyphenylglyoxylic acid (XXXIV) and flavipin (XXIX) which are related to other fungal products as described below. Nidulin and nornidulin are also benzene derivatives, and the proposed structures, XXXVIII and XXXIX, which have been put forward are of especial importance, since, if confirmed, they constitute the first recorded examples from fungi of depsidones, a group well-known in the products of lichens, which are symbionts of algae and fungi. The relationship of nidulin to known lichen products is in fact remarkably close (see below).

The biosynthesis of benzene compounds is attracting increasing attention. Light thrown on the biogenesis of the simple benzene compounds

formed by fungi may well lead to further evidence concerning the general pattern of formation of the more complex aromatic natural products. Birch & Donovan (56) have extended ideas originated by Collie in 1907 (57, 58) and developed by Robinson [see (4), p. 7] and have elaborated an "acetate hypothesis" for the biogenesis of certain phenolic and quinonoid compounds. They suggest that these substances are formed partially or wholly by the head-to-tail linkage of acetic acid units. Birch *et al.* have now presented evidence that this does in fact occur in the case of 6-methylsalicylic acid. Seshadri *et al.* have carried out a laboratory synthesis of this same mould product by "nuclear reduction" of orsellinic acid (XXII), thus following the common scheme of biogenesis which the same group have elaborated for a number of mould products (59) as well as for lichen depsides and depsidones (60). It is supposed that the C_8 unit (XXI) is derived by aldol-type condensation of a hexose and a biose; structures such as XXII or 3,5-dihydroxyphthalic acid then arise by side-chain oxidation and reduction, and the authors suggest that various known benzenoid mould products are formed from these "orsellinic units" by known processes of oxidation and reduction and condensation with other chemical units (cf. Fig. 1). This biogenetic scheme is also applied to the benzoquinone and anthraquinone series of fungal products.



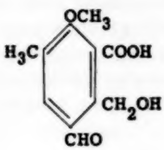
XXI XXII Orsellinic acid

FIG. 1. Biosynthesis of benzene compounds (Seshadri).

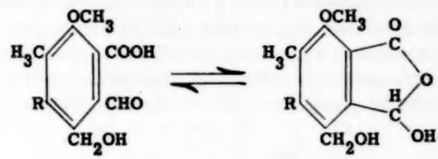
The benzene ring occurs, of course, in a large number of other fungal products mentioned in this review, e.g., cortisalin, cyclopenin, but since it is not the chief feature, these products are considered under other headings.

Gladiolic acid, dihydrogladiolic acid, and cyclopolic acid.—Dihydrogladiolic acid, from *Penicillium gladioli,* was shown by Raistrick & Ross (61) and Grove (62) to be converted on acid treatment, and even on sublimation, to the phthalide derivative (XXVI). The proposed formula (XXIII) shows the positions of the —CHO and —CH_2OH groups relative to the —COOH group. However, later evidence by Duncanson, Grove & Zealley (63) and Brown & Newbold (64) is not in agreement with this structure but rather with the alternative arrangement XXIV. Furthermore, experiments with a model compound showed that in a structure such as XXIV, a rearrangement and dehydration to XXVI does in fact take place on acid treatment or

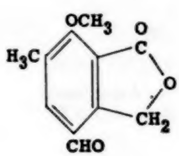
sublimation (64). Similar considerations probably apply to cyclopolic acid, a metabolite of *Penicillium cyclopium* (65), for which the new formula XXV is now put forward (63). Gladiolic acid (XXVII), also produced by *P. gladioli* (62), has now been synthesised by Brown & Newbold (66).



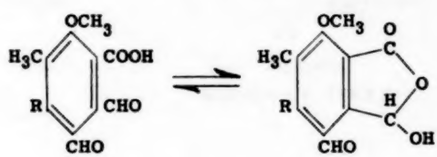
XXIII



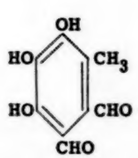
XXIV R = H, Dihydrogladiolic acid
XXV R = OH, Cyclopolic acid



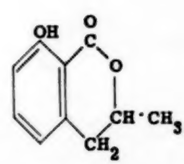
XXVI



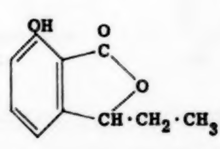
XXVII R = H, Gladiolic acid
XXVIII R = OH, Cyclopaldic acid



XXIX Flavipin



XXX Mellein



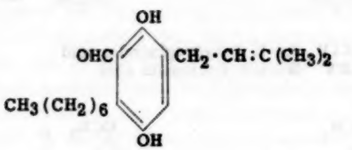
XXXI

Flavipin.—Rudman (67) has isolated a phenolic dialdehyde, flavipin, from cultures of *Aspergillus flavipes* and *Aspergillus terreus*. He has shown its structure to be XXIX and confirmed it by synthesis of certain reaction products. This compound has some similarity to gladiolic and cyclopaldic acids (XXVII and XXVIII).

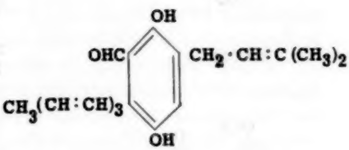
Mellein (*ochracin*).—Some doubt has existed over the exact structure of mellein, a lactone produced by *Aspergillus melleus* and *Aspergillus ochraceus* (68 to 71). The Japanese workers showed it to have the formula XXX or XXXI. That XXX is correct is shown by the synthesis by Blair & Newbold

(72, 73). Birch & Donovan (74) have pointed out that this structure accords with the acetate hypothesis.

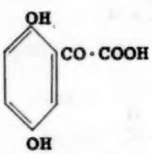
Flavoglaucin and auroglaucin.—Flavoglaucin and auroglaucin are pigments characteristic of the mycelia of the *Aspergillus glaucus* group (75). The formula for flavoglaucin put forward by Quilico *et al.* (76, 77) and quoted in these *Reviews* (1) has now been shown by the same group to be incorrect (78, 79). That flavoglaucin (XXXII) contains an aldehyde group is shown by treatment with alkaline H_2O_2 under carefully controlled conditions, when the group is replaced by OH. Panizzi & Nicolaus (80) have also removed the aldehyde group from dihydroflavoglaucin, using $AlBr_3$, to give 3-heptyl-



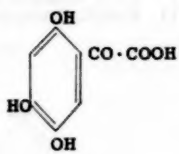
XXXII Flavoglaucin



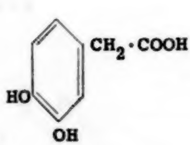
XXXIII Auroglaucin



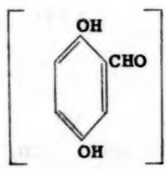
XXXIV



XXXV



XXXVI



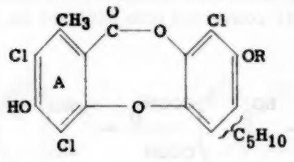
XXXVII

4-isoamyl hydroquinone, identical with a synthetic product, and have synthesized the 2,4-dinitrophenylhydrazone of a monomethyl ether of dihydroflavoglaucin. Auroglaucin can be hydrogenated to dihydroflavoglaucin (81), and a structure of the type XXXIII is proposed (78), the position of the double bonds being undetermined.

2,5-Dihydroxyphenylglyoxylic acid.—Ralph & Robertson (82) showed that *Polyporus tumulosus* produces, *inter alia*, the two acids XXXV and XXXVI when grown on a glucose medium. Moir & Ralph (83) have now shown that a third acid, 2,5-dihydroxyphenylglyoxylic acid (XXXIV), is also produced on this medium, and in larger amounts on a glycerol medium. There is a close structural relationship between XXXIV and gentisic aldehyde (XXXVII), suggested by Birkinshaw (1) as a precursor of the mould products gentisic acid, gentisyl alcohol, and possibly patulin.

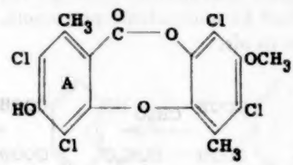
Nidulin and nornidulin (*ustin*).—A strain of *Aspergillus nidulans* has

been shown by Robertson and co-workers (84, 85) to produce two chlorine-containing compounds, nidulin, $C_{20}H_{17}O_5Cl_3$, and nornidulin, $C_{19}H_{15}O_5Cl_3$; the latter is now shown to be identical with "ustin" which was obtained by Doering *et al.* (86) from the same strain, then wrongly identified as *Aspergillus ustus*. It is, therefore, proposed that the name "ustin" be now dropped (84, 85). Degradative work leads to the probable formula XXXVIII for nidulin and XXXIX for nornidulin. Diploicin (XL), a chlorine-containing lichen depsidone, can be seen to be very similar to nidulin. Ring A, which is identical in the two compounds, is also present in another lichen depsidone,

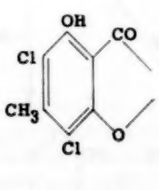


XXXVIII R = CH_3, Nidulin

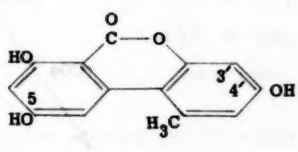
XXXIX R = H, Nornidulin



XL Diploicin



XLI



XLII Alternariol

gangaleoidin [for references, see (85)]. The moiety XLI in which the CH_3 and OH groups of ring A are interchanged, is present in the products geodin and erdin from *Aspergillus terreus* (87).

6-Methylsalicylic acid.—Birch, Massy-Westropp & Moye (88) have investigated the growth of *Penicillium griseofulvum* on glucose to which carboxy-labelled acetate was added. Analysis of the 6-methylsalicylic acid which was produced showed that labelling was in agreement with the acetate hypothesis (56).

Seshadri and co-workers (89) synthesised 6-methylsalicylic acid from orsellinic acid (XXII) by first converting the latter to the 4-tosyl derivative of the methyl ester, and then reducing with Raney nickel.

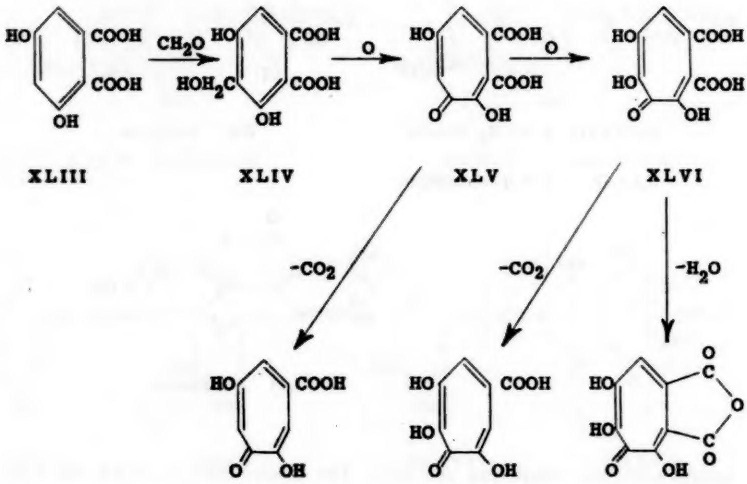
Diphenyl Derivatives

Although quinones derived from diphenyl are known in fungi (see p. 238). the sole substituted diphenyls so far reported are the lactone, alternariol, and its monomethyl ether.

Alternariol and alternariol methyl ether.—These compounds are present in the mycelia of some strains of *Alternaria tenuis*. Raistrick, Stickings & Thomas (90) proposed formula XLII for alternariol and proved its correctness by synthesis of the trimethyl ether. The position of the methoxy group of the natural monomethyl ether is probably 5 or 4′, since this ether still gives the purple ferric colour shown by alternariol itself.

Other Homocyclic Aromatic Compounds

The azulene derivatives so far observed in fungi occur only in *Lactarius deliciosus*. Evidence is now presented that the true metabolic products may in fact be nonazulenic precursors, readily converted into azulenes on exposure to air.



XLVII XLVIII XLIX

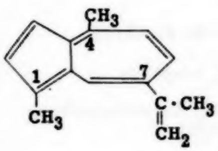
Stipitatic acid Puberulic acid Puberulonic acid

Fig. 2. Biogenesis of mould tropolones (Seshadri).

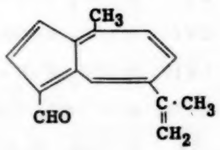
In the tropolone series, puberulic acid (XLVIII), a product of *Penicillium puberulum*, has been synthesised from stipitatic acid (XLVII), a product of *Penicillium stipitatum;* stipitatic acid was previously synthesised from 1,2,4-trimethoxybenzene by ring expansion (91). Seshadri (92) has suggested a biogenetic scheme for mould tropolones (Fig. 2) which starts from the C_8-unit XLIII, previously shown to be a possible precursor of various aromatic and quinonoid mould products (see p. 231–32).

The ring expansion stage XLIV→XLV follows the suggestions of Robinson (93, 94), while the nuclear oxidation XLV→XLVI parallels the similar reaction in the benzenoid compounds.

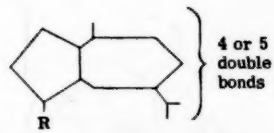
Azulenes.—Willstaedt (95, 96, 97) showed that extracts of the edible fungus *Lactarius deliciosus* gave, on chromatography, blue (lactarazulene), violet (lactaroviolin), and green (verdazulene) bands, and two orange bands. He converted the orange compound "protazulene" to lactarazulene by distillation. Benesova, Herout & Šorm (98) have found that carefully treated extracts from the fresh undamaged fungus contain practically only yellow and orange substances. The azulenes must, therefore, be regarded as artifacts.



L Lactarazulene



LI Lactaroviolin



LII R=CHO

LIII R=CH$_2$OH

LIV R=CH$_3$

Azulenogens

Lactarazulene, $C_{15}H_{16}$ (not $C_{15}H_{18}$ as recorded by Willstaedt), must possess the structural formula L, since it has been partially hydrogenated to guaiazulene (1,4-dimethyl-7-isopropylazulene), and since ozonisation yields formaldehyde (99).

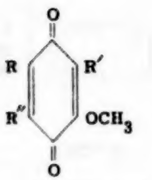
Lactaroviolin, $C_{15}H_{14}O$, is an unsaturated aldehyde, which has been completely hydrogenated to perhydroguaiazulene (100). Heilbronner & Schmid (101) show on physical grounds that the aldehyde is at position 1. Šorm and co-workers (102, 103) have converted the aldehyde to an ethyl group and reduced the exocyclic double bond to give a methylethylisopropylazulene. The two possibilities, 1-ethyl-4-methyl- and 1-methyl-4-ethyl-7-isopropylazulenes, were both synthesised, and although their properties are very similar, the balance of evidence is again in favour of the aldehyde being at position 1. Lactaroviolin therefore has the structure LI.

Indications of three yellow "azulenogenic" compounds have been obtained by Benesova *et al.* (98). Probably their structures are LII, LIII, and LIV.

Puberulic acid.—Birkinshaw, Chambers & Raistrick (104) described the bromination of stipitatic acid (XLVII) to a monobromo derivative. This compound has now been converted to puberulic acid (XLVIII) (105) in good yield, by heating with KOH [Johns, Johnson & Murray (106)].

Quinones Derived from Benzene, Diphenyl, and Terphenyl

Closely related to the benzene compounds, especially the substituted quinols mentioned above, are the substituted benzoquinones. Most of those already reported are derivatives of toluquinone (LV to LVIII), but 2,5-dimethoxybenzoquinone (LIX) has now been isolated from a culture of a higher fungus, though it may be formed simply from a quinonoid precursor in the complex medium employed (see below). Leucomelone (LXIV) is another member of the diphenylbenzoquinones produced by higher fungi and is structurally very close to polyporic acid (LXII) (107) and atromentin (LXIII) (108) [cf. also muscarufin (IV)].



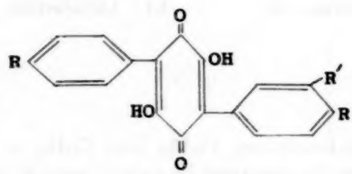
LV $R = CH_3$, $R' = R'' = H$

LVI $R = CH_3$, $R' = OH$, $R'' = H$, Fumigatin

LVII $R = CH_3$, $R' = R'' = OH$, Spinulosin

LVIII $R = R'' = CH_3$, $R' = OCH_3$, Aurantiogliocladin

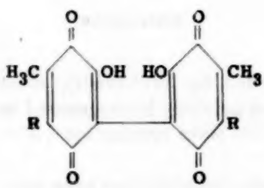
LIX $R = OCH_3$, $R' = R'' = H$



LXII $R = R' = H$, Polyporic acid

LXIII $R = OH$, $R' = H$, Atromentin

LXIV $R = R' = OH$, Leucomelone



LX $R = H$, Phoenicin

LXI $R = OH$, Oosporein

2,5-Dimethoxybenzoquinone.—Bu'Lock (109) has obtained a small quantity of this quinone (LIX) from a strain of *Polyporus fumosus* grown on a corn-steep glucose-salts medium. Since 2-methoxybenzoquinone and 2,6-dimethoxybenzoquinone have been isolated from fermented wheat germ, the possibility exists that 2,5-dimethoxyquinone is formed from a simply-related precursor in the corn-steep liquor.

Fumigatin (LVI) (110).—An interesting new synthesis of fumigatin methyl ether based on Seshadri's biogenetic scheme for mould products (59) has been carried out by Aghoramurthy, Ramanathan & Seshadri (89).

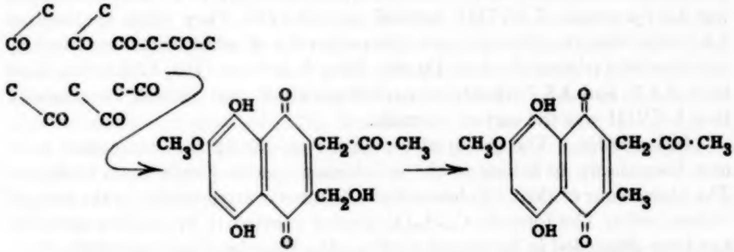
Oosporein.—Kögl & van Wessem (111) obtained this pigment (LXI) in 1944 from cultures of *Oospora colorans.* It has been isolated recently from

Chaetomium species in at least three different laboratories (112 to 115), and is closely related structurally to phoenicin (LX).

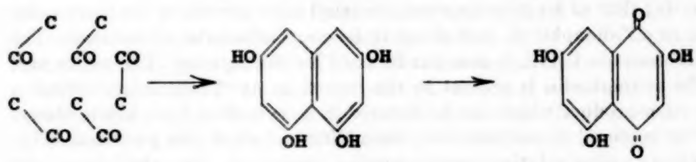
Leucomelone.—Polyporus leucomelas, an edible mushroom, was investigated by Akagi (116), who showed that it contains a brown pigment, leucomelone, which gives *p*-terphenyl on zinc dust distillation, and *p*-hydroxybenzoic acid and protocatechuic acid on oxidation with peroxide. Akagi assigned to it the formula LXIV and confirmed this by synthesis (117).

Naphthoquinones

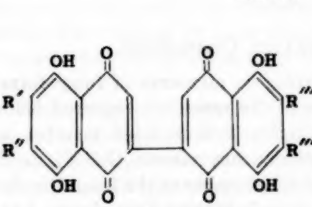
A number of substituted napthoquinones have been reported in the past from species of *Fusarium* [for references see Birkinshaw (1); Binkley (2)],



LXV Fusarubin **LXVI Javanicin**



LXVII **LXVIII Flaviolin**



R′	R″	R‴	R⁗

2-COOH, 2-CH_3, $C_8H_{12-16}O_2$

LXIX Actinorhodin

but in no case has the structure been completely established. Birch & Donovan (74) suggest that of the formulae put forward for fusarubin (? = oxyjavanicin) and javanicin (? = solanione), LXV and LXVI are the most

consistent with their acetate hypothesis. A new member of the series, flaviolin (LXVIII), has been isolated from an *Aspergillus*.

An interesting pigment, actinorhodin, isolated from *Streptomyces coelicolor*, may have a dinaphthoquinone structure. If this is confirmed, it will complete the series of diquinones, since dibenzoquinones and dianthraquinones are already established as fungal metabolic products (see p. 238 and 241).

Flaviolin.—Astill & Roberts (118) obtained, by extraction of the metabolic solution of *Aspergillus citricus*, a small yield of a red pigment which they named flaviolin. They showed that its properties were consistent with its formulation as 2 (or 3),5,7-trihydroxynaphthoquinone. Birch & Donovan (74) pointed out that their "acetate hypothesis" (see p. 231) would favour the 2,5,7-formula (LXVIII), formed via LXVIII. They have synthesised 2,5,7-trimethoxynaphthoquinone, the properties of which indicated identity with flaviolin trimethyl ether. Davies, King & Roberts (119, 120) synthesised both 2,5,7- and 3,5,7-trimethoxynaphthoquinone, and showed conclusively that LXVIII was the correct formula.

Actinorhodin.—The group of organisms producing this compound have now been shown to belong to the well-known species *Streptomyces coelicolor*. The blue colour of their alkaline culture filtrates is attributable to the salts of actinorhodin. The formula $C_{24}H_{22}O_{11}$ quoted previously in this *Review* (121) has been discarded in favour of $C_{32}H_{26-30}O_{14}$ [Brockmann *et al.* (122, 123)]. The absorption curve of the pigment is similar to that of synthetic dinaphthazarin; that of leuco-actinorhodin methyl ester acetate is similarly quite close to $\beta\beta'$-dinaphthyl, and closer to leuconaphthazarin octa-acetate. The partial formula LXIX is now put forward for the pigment. The major part of the actinorhodin is present in the mycelium as "protoactinorhodin," a reduction product which can be isolated in a crystalline form but is always slightly coloured by actinorhodin. On addition of alkali, the protoactinorhodin gives a yellow solution, rapidly turning green in air, then changing to the deep blue of the alkali salts of actinorhodin; on acidification, actinorhodin is obtained.

Anthraquinones and Related Compounds

The substituted anthraquinones occurring as pigments of fungi form a closely related group. Two new members of the series are reported below: one, asperthecin (LXXVI), is the most highly hydroxylated member isolated so far; the other, 4-hydroxy-2-methylanthraquinone (LXX), is the simplest. Raistrick reviewed the structural relationship of the fungal anthraquinones in detail in 1949 (3) and showed their derivation from chrysophanol (LXXI) which was itself shortly afterwards isolated as a mould metabolic product (124). The isolation of both chrysophanol and 4-hydroxy-2-methylanthraquinone from cultures of one organism suggests the possibility that the biosynthetic route to some of the more complex anthraquinones lies through these two compounds as intermediates. However, it seems improb-

able that the many compounds which are closely related structurally to emodin (e.g., LXXIV, LXXV) would be synthesised in the fungus by this route, involving meta-oxidation. On the other hand, emodin (LXXII) could readily arise from an acetate (polyketomethylene) precursor. Robinson (4) has pointed out that the lichen product endocrocin (LXXIII) could very easily be produced in this way, and particular interest, therefore, attaches to the isolation for the first time of endocrocin from a fungus, especially as it is accompanied by catenarin (LXXIV) and physcion (LXXV).

In two cases [chrysophanol (LXXI) and islandicin (LXXVII)], there is evidence of precursors of a different nature (see flavoskyrin, flavomycelin).

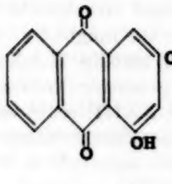
An increasing number of fungal pigments have been shown to belong to a group of dianthraquinones and similar "double molecules." Of these, only skyrin (LXXIX) has been fully characterised and its structure elucidated. Iridoskyrin (LXXX) may have a similar structure. Rugulosin and rubroskyrin are not themselves dianthraquinones but are readily converted into compounds of this type. Preliminary indications of further compounds of this series are also reported (125). Aurofusarin, isolated some years ago from *Fusarium culmorum* (126), may also belong to this group. Penicilliopsin (LXXXI) has been given a structure which relates it to the above compounds and to the plant products, hypericin (LXXXII) and sennosides A and B (127).

4-Hydroxy-2-methylanthraquinone.—Pachybasium candidum, grown on malt-peptone-glucose agar, produces a mixture of pigments, several of which appear to be anthraquinone derivatives. Shibata & Takido (128) have isolated from one fraction chrysophanol (LXXI) and 4-hydroxy-2-methylanthraquinone (LXX). This latter has already been reported as a synthetic compound but has never before been found in natural products. Identity was confirmed by several comparisons with synthetic material.

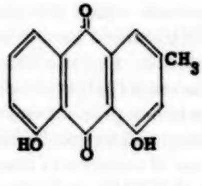
Islandicin (*funiculosin, rhodomycelin*).—Nishikawa (129) has isolated from a sterile mycelium a pigment, rhodomycelin, which he claims is identical with islandicin (LXXVII) from *Penicillium islandicum* [Howard & Raistrick (130)] and with funiculosin from *Penicillium funiculosum* [Igaraci (131)]. The identity of these latter two colouring matters has always been suspected [cf. (130)], but a direct comparison has not previously been carried out.

Endocrocin.—Shibata & Natori (132) have investigated the pigments present in a strain of *Aspergillus amstelodami*, a member of the *Aspergillus glaucus* series. In addition to flavoglaucin, auroglaucin, echinulin, and physcion, all known products of this series, they obtained catenarin (LXXIV) and endocrocin (LXXIII), previously known only as a lichen product. With the exception of boletol, this is the first fungal anthraquinone to carry a carbon substituent at any position other than C_2 and the first to have any substituent definitely established at C_3.

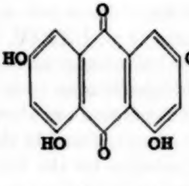
Catenarin.—This compound (LXXIV), obtained originally from *Helminthosporium* species (133), and more recently from *Aspergillus amstelodami*



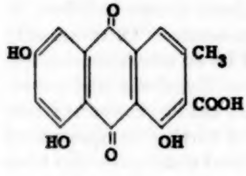
LXX



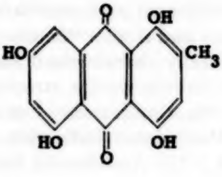
LXXI Chrysophanol



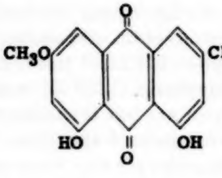
LXXII Emodin



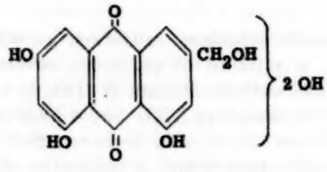
LXXIII Endocrocin



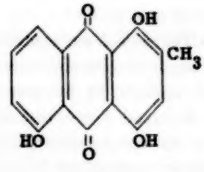
LXXIV Catenarin



LXXV Physcion



LXXVI Asperthecin



LXXVII Islandicin

(132), is a degradation product of rubroskyrin (see below), which occurs in the islandicin-producing strains of *P. islandicum* (134). Shibata, Takido & Nakajima (125) have now isolated catenarin from one of these same strains.

Asperthecin.—The colouring matter of the purple-red perithecia and ascospores of *Aspergillus quadrilineatus* has been shown by Howard & Raistrick (135) to be a pentahydroxy-2-hydroxymethylanthraquinone. The pigment, named asperthecin (LXXVI), is probably characteristic of a number of other species of the *Aspergillus nidulans* group. It can be reduced by HI and red phosphorus and reoxidised to emodin and, therefore, has the partial structure LXXVI. Chemical and spectral evidence (135, 136) concerning the positions of the two remaining phenolic groups lead to conflicting conclusions.

Flavoskyrin.—The strain of *P. islandicum* which produces skyrin (see below) also produces a crystalline yellow pigment flavoskyrin, probably $C_{15}H_{12}O_5$, which readily loses a molecule of water to yield chrysophanol (LXXI) [Howard & Raistrick (137)].

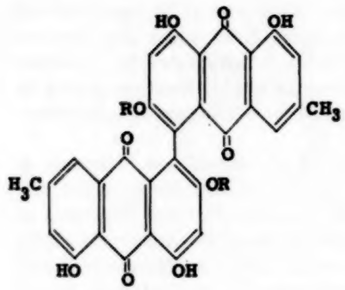
Flavomycelin.—In addition to islandicin, Nishikawa (129) has obtained from the same sterile mycelium another pigment, to which the formula $C_{15}H_{12}O_6$ is given. This substance, flavomycelin, is dehydrated by sulphuric acid to islandicin. Flavomycelin and flavoskyrin would therefore appear to belong to the same group of compounds, and flavomycelin may be a hydroxyflavoskyrin.

Skyrin (endothianin).—From a strain of *P. islandicum*, Howard & Raistrick (137) isolated a new pigment which they named skyrin (LXXIX). Shibata *et al.* (138) have described the isolation from *Endothia parasitica* of a colouring matter, endothianin, now shown to be identical with skyrin (139). Howard & Raistrick showed that skyrin was probably a "di-emodin," and this view was confirmed by Shibata *et al.* (140); in particular the latter workers showed by model compounds that this type of structure was consistent with the facile reductive cleavage of skyrin by sodium dithionite to two molecules of emodin which had been observed by Howard & Raistrick. Tanaka & Kaneko (141) have now synthesised LXXVIII and have shown that it is identical with the skyrin dimethyl ether obtained by the action of diazomethane on the pigment. Skyrin is therefore LXXIX.

Rubroskyrin and iridoskyrin.—The strains of *P. islandicum* which produce islandicin also produce a red pigment, rubroskyrin, probably $C_{30}H_{22}O_{12}$, and a red pigment with a green iridescence, named iridoskyrin (LXXX), $C_{30}H_{18}O_{10}$ (134). The latter compound is isomeric with skyrin and is formed in 82 per cent yield when rubroskyrin is treated with cold concentrated H_2SO_4. When rubroskyrin is heated in nitrogen, good yields of islandicin (LXXVII) (41 per cent) and iridoskyrin (31.9 per cent), together with smaller amounts (7.5 per cent) of catenarin (LXXIV), are obtained. For iridoskyrin, the tentative formula LXXX is put forward by Howard & Raistrick (134). One of the main objections—that iridoskyrin is not reductively split by dithionite, as is skyrin—is now removed by the finding of Shibata *et al.* (140) that 4,4′-dihydroxy-1,1′-dianthraquinone is similarly not affected by this reagent, while 2,2′-dihydroxy-1,1′-dianthraquinone is readily split.

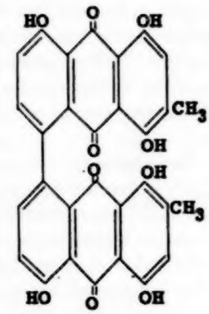
Rugulosin (radicalisin).—Breen *et al.* (142) obtained from the mycelia of strains of *Penicillium rugulosum* a strongly dextrorotatory yellow colouring matter, rugulosin. The pigment, radicalisin, isolated by Shibata *et al.* (138) from *Endothia fluens* [Shear & Stevens (= *E. radicalis* Fr.)] was shown to be identical with rugulosin (139). The compound has many properties similar to those of rubroskyrin, the differences being largely explicable by postulating that rubroskyrin contains two more hydroxyl groups than rugulosin. Thermal decomposition of rugulosin yields chrysophanol and emodin; heating with alkali also gives chrysophanol in nearly 50 per cent yield, and cold sulphuric acid converts it into aurantio-rugulosin, an optically inactive compound ($C_{30}H_{18}O_8$) with properties similar to those of iridoskyrin.

Penicilliopsin.—In 1940 Oxford & Raistrick (143) isolated from laboratory cultures of the fungus *Penicilliopsis clavariaeformis*, grown in the dark, an orange crystalline colouring matter which they named penicilliopsin

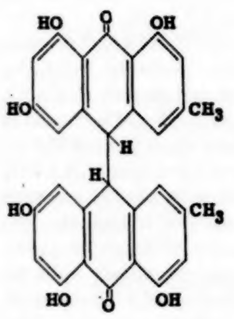


LXXVIII R = CH_3

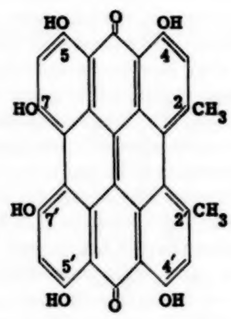
LXXIX R = H, Skyrin



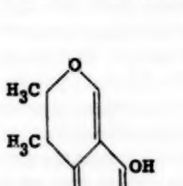
LXXX Iridoskyrin



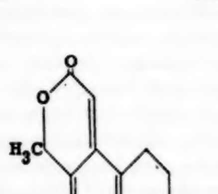
LXXXI Penicilliopsin



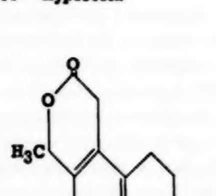
LXXXII Hypericin



LXXXIII Citrinin



LXXXIV Fuscin



LXXXV Dihydrofuscin

($C_{30}H_{24}O_8$). This pigment is readily oxidized at room temperature in air in suitable solvents to oxypenicilliopsin ($C_{30}H_{20}O_9$), solutions of which, on exposure to light, yield the isomeric irradiated oxypenicilliopsin ($C_{30}H_{20}O_9$) in chocolate brown needles; solutions of the latter have an intense red fluorescence. Penicilliopsin is clearly related structurally to some of the colouring matters just described, since, on heating alone it gives emodin anthranol, and on oxidation with nitric acid it gives tetranitroemodin. Penicilliopsin is also closely related to the photodynamically active colouring matter, hypericin, from *Hypericum perforatum*, St. John's wort, which Brockmann and his co-workers have shown to have the structure LXXXII (144, 145). As a result of this work the Brockmann school concluded that penicilliopsin has the structure LXXXI and that a fraction of the irradiated oxypenicilliopsin which they prepared by a different and more drastic method than that used by Oxford & Raistrick (143) is identical with hypericin. This conclusion is at variance with Oxford & Raistrick's findings that, although the very remarkable absorption and fluorescence spectra determined in 1939 by Dhéré & Castelli (146) on irradiated oxypenicilliopsin and hypericin were indistinguishable from each other, certain chemical tests threw doubt on the two substances being identical.

Other Quinonoid Compounds

The substituted methylene-quinone grouping is not common in fungal products. It occurs in the yellow pigment citrinin (LXXXIII) from *Penicillium citrinum* and other moulds (147, 148), and has now been shown to be the chromophoric group in fuscin (LXXXIV).

Fuscin.—This orange pigment was isolated by Michael (149) from culture filtrates of *Oidiodendron fuscum* and its chemistry studied by Birkinshaw *et al.* (150). Barton & Hendrickson have now put forward a constitutional formula, LXXXIV, which they have confirmed by the total synthesis of fuscin (151, 152). Birch (153) has also deduced the same formula on the basis of the chemical evidence already published.

It should be added that fuscin is present among the metabolic products of one strain of *O. fuscum* in the reduced form, dihydrofuscin (LXXXV), which is a trisubstituted pyrogallol derivative. This is an uncommon grouping in fungi but is present in the substituted phthalaldehyde flavipin (see p. 233).

Heterocyclic Nitrogen Compounds

Patterns are beginning to emerge among the somewhat miscellaneous collection of nitrogenous heterocyclic compounds elaborated by fungi. Several types may be distinguished, only four of which will be considered here

(*a*) Indole derivatives. These include the large group of ergot alkaloids, normally considered under the heading of plant alkaloids, though they are in fact products of fungi growing parasitically on various Gramineae. New

members of this group are described below. Echinulin (XCIV), one of several metabolic products of the *Aspergillus glaucus* series, has now been shown to contain the indole nucleus. Gliotoxin (XCVIII) (154), can be regarded as a reduced indole derivative.

(*b*) Pyrazine and reduced pyrazine derivatives. Echinulin and gliotoxin clearly belong also to this group, as does aspergillic acid (XCIX). Pulcherrimin, a yeast product, has been given a structure closely similar [cf. pulcherriminic acid (C)], though doubt is now cast on this formula. These four formulae all contain the grouping CI.

(*c*) Pyridine and quinoline group. Fusaric acid (CV) is a simple pyridine carboxylic acid and viridicatin (CII), a hydroxyquinoline compound. Cyclopenin (CIII or CIV) is closely related to viridicatin. A reduced N-methyl pyridine is also present in the ergot alkaloids.

(*d*) Purines. The antibiotic puromycin (CVI), described in detail in the 1955 review (2), bears a striking resemblance to cordycepin (CVII), isolated from a higher fungus.

Ergot.—That the ergot alkaloids owe their origin to the activities of the fungus (*Claviceps purpurea* or other *Claviceps* spp.) and not of the host plant, is further emphasised by the recent isolation of a number of new alkaloids, closely related to lysergic acid, from laboratory cultures of various ergot fungi grown on simple media. In a series of papers (155 to 166), Abe and co-workers have surveyed about 500 strains of ergot fungi on host plants of 24 species. Abe has classified them morphologically into 16 "races," some of which have been studied in detail, in particular the "Agropyrum type," found on *Agropyrum semicostatum, Trisetum bifidum, Festuca rubra*, etc., and the "Elymus type," found on *Elymus mollis*. From the natural ergots and from artifical cultures of these and other ergot fungi, Abe and co-workers have isolated no fewer than six new alkaloids, the probable structures of which are shown in Figure 3. Penniclavine (LXXXVII) was first isolated by Stoll *et al.* from artificial cultures of an ergot found on African *Pennisetum typhoideum* (167), and its structure was later proposed by the same group (168). It should also be noted that varying amounts of known alkaloids which are derivatives of lysergic acid are found alongside these new compounds, and no doubt the biosynthesis of the whole group follows a common general pattern. Abe *et al.* (163, 165) suggest an aldehyde precursor, XCII, for both lysergic acid and elymoclavine, and hence for the remaining alkaloids.

Accepting the formula XCIII for lysergic acid, the main evidence for the structures of these compounds can be summarised as follows. Elymoclavine (LXXXIX) can be reduced catalytically to a mixture of two dihydro-compounds, shown to be identical with dihydrolysergol and dihydro-isolysergol-I, two of the possible stereoisomers of structure XCI; these two alcohols are obtained by reduction of the corresponding dihydrolysergic acids (169). Compound X, isolated from various ergots and artificial cultures, is stated to be a third stereoisomer of structure XCI. Compound Y (XC), which occurs alongside compound X, is identical with the dihydro compound

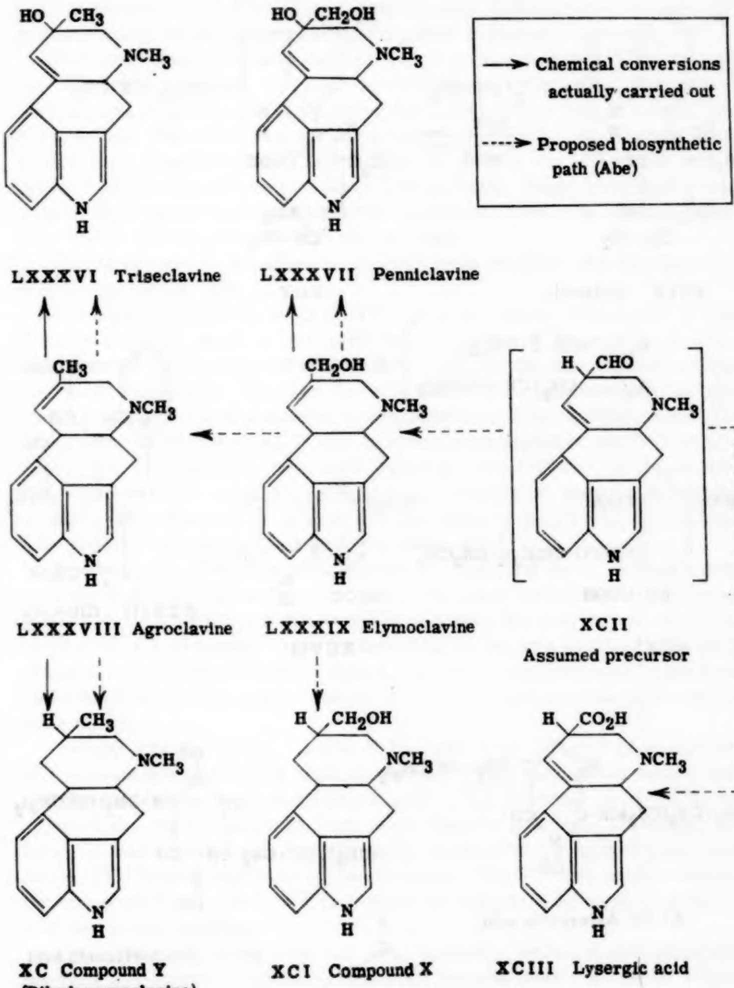
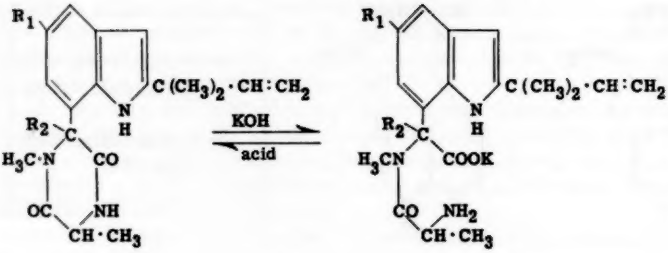


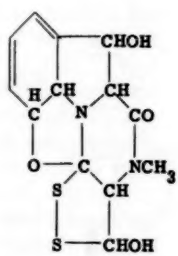
FIG. 3. Ergot alkaloids.

obtained by reduction of agroclavine. Agroclavine has not been related directly to any known ergoline derivative, but isolation of β-methylquinoline as a degradation product supports formula LXXXVIII. Both agroclavine and elymoclavine can be oxidised with dichromate and sulphuric acid and

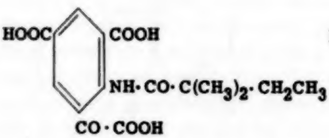


XCIV Echinulin XCV

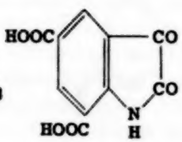
R_1, $-CH:C(CH_3)_2$
R_2, $-CH_2 \cdot CH:C(CH_3)_2$
} or vice versa



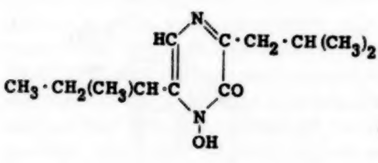
XCVIII Gliotoxin



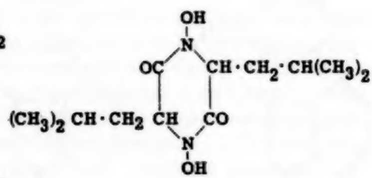
XCVI



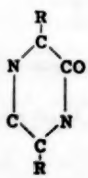
XCVII



XCIX Aspergillic acid



C Pulcherriminic acid



CI

yield triseclavine (LXXXVI) and penniclavine (LXXXVII) respectively. Penniclavine gives formaldehyde on periodate oxidation.

Stoll and co-workers have published a preliminary report (170) of three new alkaloids occurring alongside agroclavine, elymoclavine, and penniclavine. One is an isomer of penniclavine ($C_{16}H_{18}O_2N_2$) and is named isopenniclavine. The other two are an isomeric pair ($C_{16}H_{18}ON_2$) named setoclavine and isosetoclavine. They appear to contain an OH group and a double-bond in the 9,10 position (as in lysergic acid). These properties would suggest that one or other of these two compounds might be identical with triseclavine, but no comparison is yet reported.

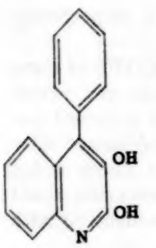
The structure of the alkaloids derived from lysergic acid has been reviewed by Glenn (171).

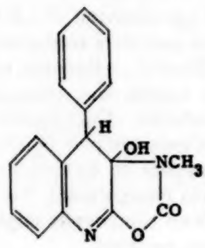
Echinulin.—Quilico *et al.* (172, 173) have continued their study of this complex product, which is obtained from cultures of *Aspergillus glaucus*, accompanied by flavoglaucin (see p. 234), auroglaucin, and various anthraquinonoid substances. The new formula XCIV has been assigned to this product on the basis of a number of degradation products. Oxidation of the hexahydro derivative of XCV with alkaline permanganate yields *inter alia* XCVI, XCVII, and isovaleric acid. Pyrolysis of XCV and the hexahydro derivative of XCV produces methylamine, ethylamine, and C_{22} indole derivatives. Hydrolysis of the hexahydro derivative of XCIV with 48 per cent HBr yields L (+)-alanine and an indole amino acid.

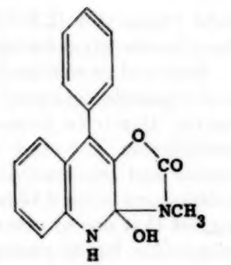
Pulcherrimin.—Pulcherrimin, a pigment produced under certain conditions by the yeast *Candida pulcherrima*, has been formulated by Kluyver *et al.* (174) and by Cook & Slater (175) as a complex iron salt of pulcherriminic acid (C). However, Cook & Slater (176) have now synthesised a compound which should be identical with C, but the properties are different from those of pulcherriminic acid. The structure of the pigment is, therefore, in some doubt.

Fusaric acid.—Yabuta, Kambe & Hayashi (177) isolated from cultures of *Fusarium heterosporum* a nitrogenous acid which they called fusaric acid and formulated as 5-*n*-butylpicolinic acid (CV). Plattner *et al.* (178) reported the same compound from other Fusaria and from *Gibberella fujikuroi;* it was also isolated, together with gibberellin A and gibberellic acid (see p. 231) from *G. fujikuroi* by Stodola *et al.* (51). Plattner *et al.* confirmed the structure by comparison with a synthetic sample. The compound causes wilt in tomato seedlings.

Cyclopenin and viridicatin.—A new, optically active mould metabolic product, cyclopenin, $C_{17}H_{14}O_3N_2$, has been isolated from the culture filtrate of a strain of *Penicillium cyclopium* by Bracken, Pocker & Raistrick (179). It is readily decomposed by acid, forming one molecule each of CO_2, methylamine, and viridicatin, $C_{15}H_{11}O_2N$. This latter substance was obtained from the mycelium of a strain of *Penicillium viridicatum* (a species closely related to *P. cyclopium*) by Cunningham & Freeman (180). It was also isolated by



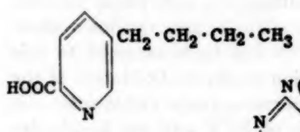




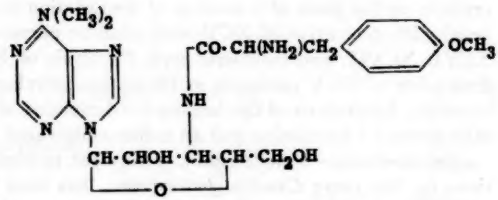
CII Viridicatin

CIII

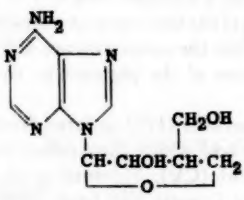
CIV

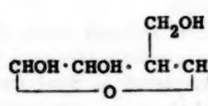


CV Fusaric acid



CVI Puromycin





CVII Cordycepin

CVIII Cordycepose

Bracken *et al.* (179) from five strains of *P. cyclopium*, but it is not present in the mycelium of the strain producing cyclopenin. Bracken *et al.* have shown the formula of viridicatin to be CII, by hydrolysis to *o*-aminobenzophenone and oxalic acid, and they have proved the structure by synthesis. For cyclopenin, either the formula CIII or CIV is proposed. Though cyclopenin is readily decomposed to yield viridicatin, it is certain that viridicatin is not an artifact arising during the isolation prodedure.

Cordycepin.—Raphael (181) has reported the synthesis of the racemic

form of the branched chain carbohydrate, cordycepose (CVIII), obtained by hydrolysis of cordycepin (CVII), a metabolite of *Cordyceps militaris* (182).

LIST OF AUTHORITIES FOR SPECIES OF MICROORGANISMS CITED IN THE TEXT

Agrocybe dura	(Bolt.) Fr.	*Fusarium culmorum*	(W. G. Smith) Sacc.
Alternaria tenuis	auct.	*Fusarium heterosporum*	Nees
Amanita muscaria	(Linn.) Fr.	*Gibberella fujikuroi*	(Saw.) Wr.
Aspergillus amstelodami	(Mangin) Thom & Church	*Lactarius deliciosus*	(Linn.) Fr.
		Nocardia acidophilus	
Aspergillus citricus	(Wehmer) Mosseray	*Oidiodendron fuscum*	Robak
Aspergillus flavipes	(Bain. & Sart.) Thom & Church	*Oospora colorans*	van Beyma
		Pachybasium candidum	(Sacc.) Peyronel
Aspergillus fumigatus	Fresenius	*Penicilliopsis clavariaeformis*	Solms-Laubach
Aspergillus glaucus	Link ex Fr.		
Aspergillus melleus	Yukawa	*Penicillium cyclopium*	Westling
Aspergillus nidulans	(Eidam) Winter	*Penicillium funiculosum*	Thom
Aspergillus ochraceus	Wilhelm	*Penicillium gladioli*	Machacek
Aspergillus quadrilineatus	Thom & Raper	*Penicillium griseofulvum*	Dierckx
		Penicillium islandicum	Sopp
Aspergillus terreus	Thom	*Penicillium notatum*	Westling
Aspergillus ustus	(Bainier) Thom & Church	*Penicillium puberulum*	Bainier
		Penicillium rugulosum	Thom
Caldariomyces fumago	Woronichin	*Penicillium stipitatum*	Thom
Candida pulcherrima	(Lindner) Windisch	*Penicillium viridicatum*	Westling
Claviceps purpurea	(Fr.) Tul.	*Polyporus anthracophilus*	Cooke
Clitocybe diatreta	Fr.	*Polyporus biformis*	
Cordyceps militaris	(Linn.) Link	*Polyporus fumosus*	(Pers.) Fr.
Corticium croceum	Bres.	*Polyporus leucomelas*	(Pers.) Fr.
Corticium salicinum	Fr.	*Polyporus tumulosus*	Cooke
Daedalea juniperina	Murr.	*Poria corticola*	Fr.
Drosophila subatrata	(Batsch) Quél.	*Poria tenuis*	Schw.
[now *Psathyrella subatrata*	(Batsch) Fries]	*Streptomyces coelicolor*	(Reiner-Müller) Waksman & Henrici
Endothia fluens	Shear & Stevens		
(=*E. radicalis*	Fr.)	*Streptomyces limosus*	Lindenbein
Endothia parasitica	(Murr.) And. et And.	*Thelephora palmata*	(Scop.) Fr.

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BIOLOGICAL OXIDATIONS[1,2]

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In this review, attention is directed chiefly to reactions in isolated systems, the detailed properties of individual enzymes and coenzymes, and the study of reaction mechanisms. The general physiological aspects of biological oxidations are appropriately considered in separate reviews of the intermediary metabolism of the major groups of compounds. The review begins with the properties and reactions of pyridine nucleotides and continues through flavoproteins, hemeproteins, and the more complex events that occur in mitochondria and chloroplasts.

The Oxidation and Reduction of Pyridine Nucleotides

Stereospecificity.—The discovery by Fisher *et al.* (1) that the reduction of DPN by ethanol in the reaction catalyzed by the yeast alcohol dehydrogenase is a stereospecific transhydrogenation has had a number of consequences. Not the least of these were the experiments of Pullman, San Pietro & Colowick (2) which led to the recognition that DPN becomes reduced in the position *para* rather than *ortho* to the pyridine ring nitrogen. Transhydrogenation and the site of reduction of the pyridine ring have been studied in a nonenzymatic system by Mauzerall & Westheimer (3). 1-Benzyl-4-deuterodihydronicotinamide transfers deuterium to malachite green while no deuterium is transferred in the reduction of the dye by the 2- and 6-deutero nicotinamide isomers. Enzymatic experiments by Loewus, Vennesland & Harris (4) with deuterium in the 2-, 4-, and 6-positions of the pyridine ring in DPNH led to similar results. The quinonoid structure of DPNH was thus confirmed by an independent method. The work of Loewus, Vennesland, and co-workers had revealed that the dehydrogenases of ethanol (1), lactate (5), and malate (6) all catalyze the transfer of deuterium from substrate to DPN to yield the same optical configurations at the site of addition. It is the deuterium and not the hydrogen in the 6-position of the DPNH produced in the above reactions that is transferred in the reverse reactions catalyzed by any of the same enzymes. However in the interconver-

[1] The survey of the literature pertaining to this review was completed in December, 1955.

[2] The following abbreviations are used in this chapter: ADP for adenosinediphosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); FAD for flavin-adenine dinucleotide; FADH for flavin-adenine dinucleotide (reduced form); FMN for flavin mononucleotide; FMNH for flavin mononucleotide (reduced form); GSH for reduced glutathione; GSSG for oxidized glutathione; TPN for triphosphopyridine nucleotide; TPNH for triphosphopyridine nucleotide (reduced form).

sion of testosterone and androstane-3,17-dione catalyzed by an enzyme from *Pseudomonas*, it was found by Talalay, Loewus & Vennesland (7) that the antipodal 6-position in DPNH is involved. Thus when deuterium has been introduced into DPNH with this enzyme it is the hydrogen and not the deuterium in the 6-position which is transferred in the reoxidations catalyzed by the first three enzymes.

Another *Pseudomonas* enzyme exhibits the same stereospecificity as the testosterone dehydrogenase from *Pseudomonas*. This is the enzyme which catalyzes the reaction:

$$\text{TPNH} + \text{DPN} \leftrightarrow \text{DPNH} + \text{TPN} \qquad 1.$$

Contrary to previous reports this reaction is now established by San Pietro, Kaplan & Colowick (8) to be a transhydrogenation with a sterospecificity on DPNH opposite to that obtained with alcohol dehydrogenase. The geometric configurations in the enzyme-coenzyme complexes should be quite different in the two cases, and it is not yet clear whether the optical specificity of the bacterial enzymes is a peculiarity of *Pseudomonas* or whether it is a steric requirement in the formation of a ternary complex between the proteins, the nucleotide, and another large hydrogen donor or acceptor. The DPN enzyme from *Echeridia freundii* which catalyzes the oxidation of 3-α-hydroxycholanic acid to 3-ketocholanic acid [Hayaishi and co-workers (9)] should be examined in this respect.

The glyceraldehyde-3-phosphate dehydrogenases of yeast and rabbit muscle have been studied by Loewus, Levy & Vennesland (10) in reactions run in D_2O. In both cases very little deuterium was found in the DPNH. However the amount of deuterium from the water which did enter the DPNH, although small, was larger than had previously been observed with other dehydrogenases. Prior to the transhydrogenation work Hayes (11) had incubated the mammalian enzyme in the absence of substrate with unlabeled DPNH and P^{32}-labeled DPN (12). After separation of the two forms of the nucleotide all of the radioactivity was found to be in the DPN and none in the DPNH. This was interpreted at the time as indicating that the enzyme did not mediate "electron transfer" between DPN and DPNH. Similar results were obtained by Kaplan and co-workers (13) with C^{14}-labeled DPN and the alcohol, glyceraldehyde-3-phosphate, and lactate dehydrogenases. None of these enzymes appeared to undergo oxidation reduction cycles in the catalytic process. The same conclusion could be reached from the fact that high concentrations of alcohol dehydrogenase did not oxidize added DPNH or reduce DPN in mixtures which did not contain substrate [Hayes & Velick (14)].

Some years ago it was observed by Cori, Velick & Cori (15) that pyruvate reduction by DPNH and lactic dehydrogenase was stimulated by the presence of high concentrations of glyceraldehyde-3-phosphate dehydrogenase which itself had no lactic dehydrogenase activity. Most of the DPNH should have been bound by the glyceraldehyde enzyme under these condi-

tions, and the possibility was suggested that the lactic dehydrogenase reacted directly with the DPNH bound to the other enzyme, a process of some interest in terms of organized reaction sequences. More recently Mahler & Elowe (16) reached similar conclusions from the DPNH-cytochrome-*c* reductase reaction carried out in the presence of high concentrations of the glyceraldehyde-3-phosphate dehydrogenase. However, studies of DPNH dissociation from the glyceraldehyde enzyme (12) and several instances of activation effects by proteins and salts weaken the interpretation of the kinetic results. If two dehydrogenase proteins were to bind and activate the same molecule of DPNH serious geometric problems would arise unless they acted on opposite sides of the pyridine ring. The stereospecificity of the glyceraldehyde-3-phosphate dehydrogenase with respect to the DPNH has not yet been reported.

Kaplan *et al.* (17) have isolated a new DPN isomer from highly purified preparations of DPN after destroying the "normal" isomer with *Neurospora* diphosphopyridine nucleotidase. Convincing chemical and enzymatic analyses localize the difference in the α,β isomerism of the bond joining the ribose to the pyridine ring nitrogen. Thus the specific rotation of the normal isomer in water is -34.8 and of the new isomer is $+14.3$. The specific rotation of the normal nicotinamide mononucleotide is -38.3 whereas that obtained from the isomeric DPN has a specific rotation of $+58.2$. Both mononucleotides yield the same ribose-5-phosphate, and both DPN isomers yield the same 5′-adenylic acid. Since the new α-isomer constitutes 10 to 15 per cent of commercial DPN preparations and has specific biological properties its detection is of considerable importance. Isomerization during isolation appears to be excluded.

Zinc in pyridine nucleotide specific dehydrogenases.—In 1951 Nason, Kaplan & Colowick (18) observed that a number of enzymes in *Neurospora* were increased in concentration or were unaffected when the organism was grown in a zinc-deficient medium. The concentration of alcohol dehydrogenase, however, decreased precipitously under such conditions. Valee & Hoch (19)have now found by spectrographic analysis of several crystalline preparations that yeast alcohol dehydrogenase contains four atoms of zinc per molecule in agreement with the stoichiometry of DPNH binding observed by Hayes & Velick (14). The enzyme is inhibited by chelating agents such as *o*-phenanthroline and is not reactivated by the addition of zinc salts. The authors believe that the zinc remains attached to the inhibited enzyme and that the inhibition by *o*-phenanthroline is competitive with DPN. Following this work Theorell, Nygaard & Bonnichsen (20) found one atom of zinc per DPNH binding site in the horse liver alcohol dehydrogenase and pictured the zinc as linking the carboxamide group of DPN to the protein. The crystalline glutamic dehydrogenase of liver, which utilizes DPN, has also been found by Valee, Adelstein & Olson (21) to contain zinc as an essential constituent. The zinc content of this protein reaches a maximum of four to five atoms per molecule, molecular weight one million, after three

crystallizations. Inhibition by chelating agents is slow and requires preincubation of the enzyme with the inhibitor.

The kinetics of alcohol dehydrogenase.—In kinetic studies of the reaction catalyzed by alcohol dehydrogenase crystallized from horse liver Theorell & Chance (22) accounted for the results with a reaction scheme which explicitly considered enzyme-coenzyme complexes but took no account of intermediary complexes that might be formed between the enzyme and alcohol or aldehyde. Alberty's analysis of the Michaelis constants, maximal velocities, and equilibrium constants obtained by these workers led to somewhat anomalous results (23). Theorell, Nygaard & Bonnichsen (20) have reinvestigated the kinetics of the reaction using a recording fluorimeter [Theorell & Nygaard (24)] which was much more sensitive than a spectrophotometer. The revised data were more closely in accord with the Haldane relation derived by Alberty for the mechanism originally proposed by Theorell & Chance:

$$K_{eq} = V_f^3 K_{DPNH} K_{ALD} / V_r^3 K_{DPN} K_{ALC} \qquad 2.$$

where V_f and V_r are maximal velocities in the forward and reverse directions respectively, K_{eq} is the equilibrium constant for the over-all reaction, and K_{DPNH} and K_{DPN} are Michaelis constants. None of the Michaelis constants represent dissociation constants of the corresponding complexes. Although data presented by Theorell & Nygaard do not seem to require consideration of intermediary complexes of enzyme with alcohol and aldehyde, they point out that such complexes might occur but not be revealed in rate limiting steps. On the basis of inhibitions by hydroxylamine (25) and a hydroxyl ion-catalyzed addition of hydroxylamine to the pyridine ring of DPN, Burton & Kaplan (26) propose ternary enzyme-coenzyme-substrate complexes as intermediates in the reactions catalyzed by both the liver and yeast alcohol dehydrogenases. In particular these workers postulate an intermediary addition compound between alcohol and the pyridine ring of the bound DPN. Some sort of ternary complex seems obligatory to account for the facts that the enzyme itself does not undergo reversible oxidation-reduction cycles in the reaction and that the reaction is a transhydrogenation.

The reaction catalyzed by the yeast enzyme behaves as though ternary substrate-enzyme-coenzyme compounds were formed as intermediates with independent binding of substrate and coenzyme by the protein [Hayes & Velick (14)]. Moreover the dissociation constants of the DPN and DPNH protein compounds, determined by the ultracentrifugal separation method and checked by equilibrium shifts in the over-all enzymatic reaction at high enzyme concentration, agree approximately with the corresponding Michaelis constants, K_{DPN} and K_{DPNH}. This relation does not occur as a general case in the mechanisms for "two substrate" reactions considered by Alberty (23). Gierer (27) has studied the effects of temperature on the reaction catalyzed by the yeast enzyme and has concluded that the entropies of activation and association of both substrates and products are small, a result which suggests to him that the reactants are bound in a rigid manner to the surface

of the enzyme. Sizer & Gierer (28) from a study of pH effects conclude that the hydrogen ion which appears as a substrate or product in the reaction is donated or accepted by a polar group on the enzyme which is characterized by a dissociation constant of about 10^{-7} *M*. Johannesmeier & Redetzki (29) have prepared an antibody to the yeast alcohol dehydrogenase which inhibits the yeast enzyme but not the horse liver enzyme.

A theoretical treatment of pH and salt effects in enzymatic reactions by Alberty (30) and application of such theory to the fumarase reaction by Frieden & Alberty (31) illustrate what may be learned about enzymatic reactions of all types by a thorough kinetic analysis over a wide range of conditions.

Glyceraldehyde-3-phosphate dehydrogenases.—Hageman & Arnon (32) report the purification of a glyceraldehyde-3-phosphate dehydrogenase from pea seeds which equals the specific activity of the enzyme crystallized from muscle. This enzyme is DPN specific and occurs in seeds and in seedlings germinated in the dark. The TPN specific enzyme previously discovered by Arnon (33) in green plant tissues is found by Hageman & Arnon (34) to appear only in the green shoots of seedlings exposed to light. Seedlings germinated in the dark develop this activity upon exposure to light. Rosenberg & Arnon (35) have now partially purified a third glyceraldehyde-3-phosphate dehydrogenase activity from green leaves. This one is TPN specific. Unlike the other two enzymes it does not catalyze an oxidative phosphorylation. The reaction is in fact stated to be inhibited by phosphate and arsenate, and the product in the absence of these ions has been identified as 3-phosphoglyceric acid. It is not yet clear whether this activity represents a physiological function of a single leaf protein.

Although the existence of thiol functions and the occurrence of an acyl-enzyme intermediate in the reaction catalyzed by the mammalian glyceraldehyde-3-phosphate dehydrogenase have been established, the evidence that the intermediate is a thio ester is still circumstantial [Velick (36), Boyer & Segal (36)]. Krimsky & Racker have summarized their attempts to establish glutathione as the carrier of the essential thiol groups of the enzyme (37). The approach to this problem is difficult and indirect because the purified enzyme has not been reversibly inactivated and reactivated by the removal and addition of glutathione. The enzyme may be acetylated by incubation with acetyl phosphate, and the idea that a catalytic site is involved receives some support from the observation that the acetyl-enzyme is stabilized by the removal of the bound DPN. From the isolated enzyme, so treated, an acetyl hydroxamic acid can be formed chemically. Tryptic digests of the enzyme contain glutathione activity in enzymatic tests, and this activity is not present in digests of the acetylated enzyme. These observations suggest that the intermediate in the catalytic reaction is some bound form of S-acyl-glutathione. It should be noted that in those cases where a supposed intermediate enzyme-substrate compound has yielded to analysis, the group acceptor has been found to be some small cofactor molecule bound to the en-

zyme. However, glutathione may be bound nonspecifically by the dehydrogenase, and the occurrence of enzymatic and nonenzymatic acyl transfer reactions can confuse the results. Evidence that the peptide chains of the protein actually contain γ-glutamyl cysteinyl glycine sequences is still rather tenuous. Ambiguities in the interpretation of thiol functions of enzymes have been discussed (36). A new and possibly pertinent case of acetyl enzyme formation has been presented by Balls & Aldrich (38). These workers have acetylated chymotrypsin by incubating it with acetyl esters of various nitrophenols. Acetyl hydroxamic acid could be formed chemically from the enzyme so inactivated. Chymotrypsin has not been found to contain free thiol groups.

The molecular weight of the mammalian glyceraldehyde-3-phosphate dehydrogenase prepared in versene has been found by light-scattering measurements to be 140,000 [Dandlicker & Fox (39)]. The enzyme from yeast contains two C-terminal methionine groups [Halsey & Neurath (40)]. Inhibition of the yeast enzyme by methyl mercury nitrate is quite different from that by parachloromercuribenzoate [Halsey (41)]. Warburg and coworkers (42) have discussed objections to the acyl-enzyme intermediate theory insofar as it applies to the glyceraldehyde-3-phosphate dehydrogenase from yeast. Definitive experiments on the mechanism of action of the yeast enzyme have not yet appeared.

Isocitric dehydrogenase.—DPN and TPN specific isocitric dehydrogenases occur in plants and animals. Moyle & Dixon (43) have purified the mammalian TPNH enzyme to a point which they consider 90 to 95 per cent pure. Their results indicate that the dehydrogenase activity and the oxalosuccinate decarboxylase activity are carried by the same protein. It will be recalled that the DPN specific enzyme which catalyzes the oxidative decarboxylation of isocitrate gave no evidence of an oxalosuccinate intermediate [Kornberg & Pricer (44); Plaut & Sung (45)]. Ramakrishnan & Martin (46) find DPN and TPN isocitrate dehydrogenase activities in *Aspergillus niger* and in agreement with work on the enzymes from other sources, adenosine-5-phosphate is a cofactor for the DPN enzyme. An effect of adenosine-3-phosphate on the reversibility of the TPNH-DPN transhydrogenase of *Pseudomonas* has been attributed by Kaplan *et al.* (47) to its action as a competitive antagonist of inhibitory effects of TPN and also to its action as a specific enzyme activator.

The formation of α-ketoglutarate in a particle free extract of human term placenta is found by Villee & Gordon (48) to be stimulated by the addition of estradiol. The increased α-ketoglutarate production occurs with citrate, isocitrate, and *cis*-aconitate as substrates but not with oxalosuccinate. Experiments with fluorocitrate seem to rule out aconitase as the site of reaction of the hormone. The presence of DPN was found to be essential in aerobic experiments. The results suggest that estradiol acts upon the DPN specific isocitric dehydrogenase in this system [Gordon & Villee (49)].

DPN potential and glycollate dehydrogenase.—The full details of Rodkey's

potentiometric titration of DPN have now appeared (50). In agreement with the equilibrium data of Burton & Wilson (51) the E_0' at pH 7.0 is -0.318 volts at 30°C. In the pH range 6.5 to 10.5 the potentials are described by the relation:

$$E_h = -0.1071 + 0.0301 \log([DPN]/[DPNH]) - 0.0301 \text{ pH} \quad 3.$$

The enzyme which catalyzes the oxidation reduction equilibrium between DPN and DPNH with glycollate and glyoxalate has been crystallized from tobacco leaves by Zelitch (52) . From the equilibrium of this reaction and the potential of the DPN/DPNH couple Zelitch calculates that E_0' for the glycollate/glyoxalate pair is -0.087 volts at pH 7.0, 25°C. This is an unusually high potential for a substrate reacting with DPN in a freely reversible reaction. Since a glycollate oxidase flavoprotein occurs in plants, it was suggested that glycollate and glyoxalate form a cyclic carrier system between DPNH and oxygen:

$$H^+ + DPNH + \text{Glyoxalate} \leftrightarrow \text{Glycollate} + DPN \quad 4.$$

$$\text{Glycollate} + FAD \leftrightarrow \text{Glyoxalate} + FADH_2 \quad 5.$$

Wilson & Calvin (53) have found that the glycollate concentration increases in green plants when the carbon dioxide pressure is lowered. They believe that the glycollate arises as the two carbon fragment in the transketolase reaction and is liberated from a glycolyl-enzyme compound by hydrolysis when carbon dioxide deprivation cuts down the production of the normal acceptor, glyceraldehyde-3-phosphate.

Fatty acid synthesis.—Hele & Popjak (54) obtained the synthesis of higher fatty acids from acetate in a soluble fraction of mammary gland. The active system required the addition of DPNH, CoA, ATP, and magnesium ions. DPNH utilization paralleled fatty acid synthesis. The difficulty in reversing the CoA-linked sequence of fatty acid oxidation in mitochondria led Langdon (55) to examine a soluble liver fraction for this activity. He reports that 1-C^{14}-acetate is readily converted to higher fatty acids in a dialyzed soluble fraction of liver in the presence of DPN, TPN, and CoA, at rates comparable with those in whole homogenates. The TPNH requirement is a new one and has been localized in part to a TPNH crotonyl CoA reductase. The suggestion is made that although oxidations are favored in mitochondria, reductive syntheses may occur most readily in the reducing environment of the cytoplasm.

Glucose oxidation.—A new form of glucose oxidation has been found by Strominger *et al.* (56). Evidence had accumulated that uridinediphosphoglucuronic acid was an intermediate in the formation of glucuronides [Storey & Dutton (57)]. Strominger and co-workers discovered that this compound was made in the following reaction:

$$\text{UDPGlucose} + 2\ DPN \rightarrow \text{UDPGlucuronic acid} + 2\ DPNH + 2\ H^+ \quad 6.$$

The reaction is catalyzed by an enzyme obtained in soluble form from liver acetone powders. No evidence could be obtained for the action of two en-

zymes in the reaction nor could any compound be detected at the aldehyde oxidation level.

The glucose oxidase of liver which catalyzes the reaction,

$$\beta\text{-glucopyranose} + \text{DPN} \leftrightarrow \delta\text{-glucuronolactone} + \text{DPNH} + \text{H}^+ \qquad 7.$$

[Strecker & Korkes (58)] has been studied by Loewus and co-workers (10) in reactions run in D_2O. As in other reactions of this type the substrate is oxidized by a transhydrogenation to DPN. An improved isolation of glucose-6-phosphate dehydrogenase from brewers yeast has been worked out by Glaser & Brown (59) who have characterized some of the kinetic properties of the enzyme and have studied the equilibrium. Kelley *et al.* (60) have found unusually high concentrations of glucose-6-phosphate dehydrogenase in adrenal cortex, a tissue which has an active nonglycolytic pathway of glucose metabolism. The pathway of TPNH oxidation in this tissue should be of unusual interest.

Other dehydrogenases.—Strittmatter & Ball (61) have obtained from beef and chicken livers a DPN-specific formaldehyde dehydrogenase which has a specific requirement for glutathione. Kinetic evidence suggests that the enzyme attacks the hemimercaptal, S-hydroxymethylglutathione, but S-formylglutathione was not detected as an intermediate in the formation of formic acid. Black & Wright (62, 63, 64) describe the preparation of three enzymes from liver which catalyze the following reactions of aspartic acid and its derivatives:

$$\text{Aspartic acid} + \text{ATP} \leftrightarrow \beta\text{-Aspartylphosphate} + \text{ADP} \qquad 8.$$

$$\beta\text{-Aspartylphosphate} + \text{DPNH} + \text{H}^+ \leftrightarrow \text{Aspartic-}\beta\text{-semialdehyde} + \text{DPN} \qquad 9.$$

$$\text{Aspartic-}\beta\text{-semialdehyde} + \text{DPNH} + \text{H}^+ \leftrightarrow \text{Homoserine} + \text{DPN} \qquad 10$$

Yann & Gilvarg find that the reduction of 5-dehydroshikimic acid to shikimic acid in the pathway of aromatic amino acid synthesis in *E. coli* is catalyzed by a TPNH specific enzyme which has been partially purified (65). The reduction of TPN and DPN by molecular hydrogen in a soluble extract of *Clostridium kluyverii* is described by Korkes (66). A heat stable, alkali labile cofactor is involved in this reaction.

Strominger & Lowry (67) have developed micromethods for the assay of malate, lactate, and glutamate dehydrogenases in 3 μg. (dry weight) of serial sections through various cellular layers of brain. In Ammon's horn lactic dehydrogenase distribution parallels that of aldolase and is high in non-myelinated fibers and dendrites while the glutamate dehydrogenase occurs chiefly in the myelinated fibers. Three types of fiber tracts are distinguished by these methods. Of particular interest is the observation that retinal ganglia are rich in lactic dehydrogenase and poor in malate dehydrogenase while the reverse is true in the deeper structures. It is suggested that the high glycolytic and respiratory rates in retina are probably expressions of different metabolic processes in different cells.

A method for the reversible inactivation and reactivation of α-ketoglutar-

ate dehydrogenase by the removal and addition of thioctic acid is described by Seaman & Naschke (68). Synthesis of the optical antipodes of thioctic acid has been achieved by Walton and co-workers (69).

Flavoproteins

Although there is continued progress in the study of flavoproteins many troublesome problems confront workers in this field. Flavoproteins are usually studied in systems in which DPN, a dye, a cytochrome, or molecular oxygen are employed as the ultimate acceptors of hydrogen or electrons. Problems of acceptor as well as substrate specificity thus arise, and the specificity or lack of it may be a source of confusion. The recognition of metal ion functions in flavoproteins has introduced new variables in the acceptor problem and has greatly increased the scope of rationalizations. The feeling sometimes arises that the activities exhibited in various isolated flavoprotein systems may not be representative of optimal functions of the enzymes and in some cases may not even represent a function of physiological significance.

Xanthine dehydrogenase (*oxidase*).—This enzyme, which may be prepared in highly active form from milk and from liver, illustrates problems that arise in the study of flavoproteins. Preparations from the two sources are not identical in their catalytic properties. Incomplete reduction of the bound flavin in the milk enzyme by xanthine and an atypical spectrum originally led Ball (70) and others to search for a second prosthetic group in addition to the FAD known to be present. From kinetic data Morell (71) concluded that the incomplete reduction was attributable to the presence of inactive flavoprotein. Since no flavin reduction could be observed with DPNH as substrate he concluded that the DPNH-cytochrome-*c* reductase activity was the result of a contaminating enzyme. Mackler, Mahler & Green (72) considered the incomplete reduction of the flavin by xanthine to represent an equilibrium. On the basis of this assumption they concluded that the standard potential, E_0', of the enzyme was below that of the hydrogen electrode at pH 7, a condition which might arise, they suggested, from interaction of the flavin with the newly discovered molybdenum in the enzyme. Detectable reduction of the flavin by DPNH under such conditions might then not be expected. The potential of the enzyme is crucial in these arguments, and the usual criteria should be applied to determine whether an oxidation reduction equilibrium between the enzyme and xanthine or hypoxanthine is actually established, and if so, what the equilibrium constant is. Removal of the bound molybdenum by dilute ammonia destroyed the ability of the milk enzyme to react with cytochrome-*c*, although it did not affect reduction of dyes nor did it affect the characteristic spectrum. In line with their ideas about other metalloflavoproteins, Mackler *et al.* proposed that the molybdenum functioned by mediating reduction of a one electron acceptor.

Recently Avis *et al.* (73, 74) crystallized xanthine oxidase from milk. Although the crystallization did not result in a higher specific activity for xanthine oxidation it involved new fractionation steps which affected other

properties of the preparation. The crystalline but still inhomogeneous enzyme contained flavin, molybdenum, and also iron in the ratio 1:1:4 respectively. The activity of the enzyme with DPNH was quite low compared with that of the amorphous enzyme of the Wisconsin group.

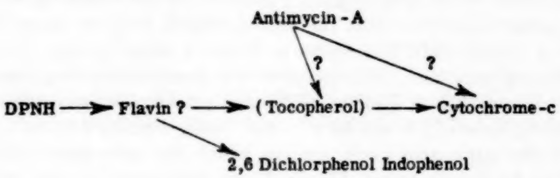
Kielley (75) has purified xanthine oxidase from calf liver. Like the milk enzyme the liver preparation contains molybdenum and iron, the ratio FAD:Mo:Fe being approximately 0.9:1:4. Oxidation of DPNH and xanthine catalyzed by this enzyme were additive, indicating that different catalytic sites and possibly different proteins were involved. Moreover DPNH was not oxidized at an appreciable rate when oxygen or cytochrome-*c* was the acceptor but was oxidized readily when the acceptor was 2,6-dichlorophenol-indophenol. Apparently neither the molybdenum nor the iron in this enzyme mediated reduction of the one electron acceptor. Morell (76) has prepared a crude xanthine oxidase from liver which catalyzes the reduction of DPN as rapidly as the reduction of methylene blue and both of them much more rapidly than the reduction of cytochrome-*c* or oxygen. He suggests that the enzyme be called xanthine dehydrogenase and that DPN is the natural acceptor. Obviously more work has to be done before the various activities and prosthetic group functions of both the milk and liver enzymes can be unravelled with confidence.

Cytochrome reductases.—The major pathway of hydrogen or electron transfer from primary acceptors to oxygen is believed to consist, in part, of an organized cytochrome sequence in mitochondria. Of this group of carriers only cytochrome-*c* has been available for direct tests as an acceptor in purified soluble systems. Thus Horecker (77) and Vernon and co-workers (78) isolated highly active flavoproteins which catalyzed the reduction of cytochrome-*c* by TPNH and DPNH respectively. Mahler & Elowe (79) found that the DPNH enzyme from heart particles contained four atoms of non-heme iron per flavin group and provided evidence which suggested that the iron mediated electron transfer from flavin to cytochrome.

DPNH-microsomal cytochrome (*cytochrome*-b′, -m, *or* -b_5) *reductase.*—Of the DPNH-cytochrome-*c* reductase activity of liver 58 per cent is in the submicroscopic particles or microsomal fraction [Hogeboom (80)]. Strittmatter & Velick (81) have isolated part of this activity as a DPNH-specific enzyme that is inert with cytochrome-*c* as acceptor but which reduces the purified microsomal cytochrome rapidly. Since the microsomal cytochrome reacts spontaneously with cytochrome-*c* it completes a DPNH cytochrome-*c* reductase system that involves a cytochrome to cytochrome electron transfer. Because of the speed of the cytochrome to cytochrome reaction the microsomal cytochrome remains predominantly in the oxidized form during the reaction, becoming completely reduced only when most of the ferricytochrome-*c* is reduced. In the absence of other information such steady state behavior in an oxidizing system might lead one to suppose that the *b* type cytochrome was not in the main pathway of electron transport. In highly active but still impure form the microsomal cytochrome reductase

contains flavin, the characterization of which as the functional group is still incomplete. Assuming a flavin to be the prosthetic group the turnover of the enzyme is about 5500 per min. at 26°C., pH 8.0. The activity of the enzyme with the microsomal cytochrome is inhibited by metal ion binding agents, but its high activity toward ferricyanide is unaffected. Methylene blue is inert as an acceptor, but dyes with potentials above or below that of methylene blue are active. In agreement with the observations of Chance & Williams (82) on the unfractionated particles the reductase has a high affinity for DPNH. When the DPNH concentration is limiting the reaction follows a zero order rate law to completion. The particles also yield a soluble TPNH cytochrome reductase which is sluggish with the microsomal cytochrome, reduces ferricyanide not at all, and is very active with methylene blue and cytochrome-*c*.

Effects of tocopherol.—A noteworthy contribution to the study of DPNH cytochrome-*c* reductase systems has been made by Nason & Lehman (83). These workers have obtained, from muscle and other tissues, particulate and solubilized enzyme preparations which catalyze the reduction of cytochrome-*c* by DPNH. The cytochrome reductase activity, which is antimycin-sensitive, disappears when the solutions are extracted with isooctane and is restored by adding back the concentrated extract or by addition of free α,β, or γ-tocopherol. No vitamin E activity could be detected in the enzyme or in the extracts, and chemical tests for tocopherols were negative. The particulate preparation also contained a succinate cytochrome-*c* reductase activity which exhibited the same behavior with tocopherols. Stimulation by tocopherols was not observed with the purified DPNH cytochrome-*c* reductase from heart particles. The authors tentatively summarize their results as follows:



11.

The possible role of tocopherols in electron transport has been recognized for some time. The present work although not decisively identifying a tocopherol as a participant in the transport chain implicates a tocopherol or some closely related lipide-soluble factor. In more complex systems the behavior of tocopherol is complex. Thus Rosencrantz (84), for example, has reported a significant increase, and not a decrease, in the Q_{O_2} of tissues from vitamin E-deficient animals in agreement with numerous older observations.

The old yellow enzyme.—Although the status of this flavoprotein from yeast is uncertain, it is of great historical importance in the study of protein coenzyme interactions. Early experiments of Theorell (85) indicated that

the enzyme had a sluggish TPNH cytochrome-*c* reductase activity. Theorell & Nygaard (24, 86) reopened investigation of coenzyme binding by apoprotein using a fluorescence recorder for the kinetic study of complex formation and for the measurement of dissociation equilibria. The second order rate constant for complex formation was determined directly as was the equilibrium constant. Anions of strong acids, particularly divalent anions, were found to promote dissociation. Vestling (87) has reinvestigated the potential of the enzyme and reports that E_0' at pH 7.0 is -0.122 volts and not -0.059 volts as previously reported by Kuhn & Boulanger (88). The new value is much closer to that for a free flavin nucleotide. The E_0'/pH slope is -0.06 in the pH range 4.8 to 8.9.

Succinic dehydrogenase.—This enzyme was traditionally inseparable from a mitochondrial matrix and was, therefore, much studied in various particulate forms. Singer & Kearney (89), however, have now obtained the enzyme in a purified soluble form by fractionation of simple alkaline extracts of mitochondrial acetone powders. Since the enzyme in this state does not react with the usual acceptors, its solubilization by such methods had previously escaped detection. It was found that phenazine methosulfate provided a link to molecular oxygen in a manometric test which served for assay and a guide to purification. The enzyme has a molecular weight of 140,000 from sedimentation velocity and diffusion, contains one to two atoms of iron per molecule, and no detectable heme (90). The activity is proportional to the iron content, and the iron is rather firmly bound. Chelating agents with high affinities for ferrous and ferric ions do not inhibit the enzyme. *o*-Phenanthroline forms a red complex with the enzyme without inhibiting it, and the color is bleached by succinate. The enzyme itself has a yellow color, and the difference spectrum, oxidized minus reduced, is typical of flavoproteins. Proteolytic digestion is necessary for release of the flavin (91), and recent tests show that the released flavin is in the form of FAD attached to a peptide (92). The results of Singer & Kearney appear to settle the long debated question as to whether or not succinic dehydrogenase is a flavoprotein. Green, Mii & Kohout (93) have prepared a particulate form of the dehydrogenase which was 60 per cent lipide, contained no other dehydrogenase of the citric acid cycle, and in which the only detectable heme was stated to be cytochrome-*b*. Flavin, heme, and nonheme iron occurred in the ratio 1:4:16, and 10 to 30 per cent of the flavin was FAD. Only 80 per cent of the heme could be reduced by succinate, and the ferrocytochrome-*b* was reoxidized by added cytochrome-*c*. These results suggest that cytochrome-*b* may be the natural acceptor of reducing equivalents from the dehydrogenase flavoprotein. Kearney, Singer & Zastrow (94) report that the soluble succinic dehydrogenase prepared in the absence of phosphate exhibits a specific requirement for phosphate. Further developments on this point are awaited with interest since similar involvements of phosphate in the action of xanthine oxidase (72), and DPNH-cytochrome-*c* reductase (79) are now being reconsidered.

TPNH glutathione reductase.—This type of enzyme activity has been detected in preparations from several plant and animal sources, but in no case was a prosthetic group of the enzyme identified. Asnis (95) has prepared a partially purified enzyme from *E. coli* which exhibits a flavoprotein spectrum and which can be resolved by acid precipitation and reactivated by FAD. The stoichiometry of the reaction was

$$TPNH + H^+ + GSSG \rightarrow TPN + 2\ GSH \qquad 12.$$

Racker (95) has purified and studied the glutathione reductases of yeast and beef liver, and they were active with both DPNH and TPNH.

Other flavoproteins.—Sutton has reported further purification of the lactic oxidase of *Mycobacterium phlei* (96). The enzyme exhibits one electrophoretic boundary. No flavin was detected spectrophotometrically, but reactivation experiments suggested that FMN was the prosthetic group. Zucker & Nason (97) describe a five- to tenfold purified enzyme from *Neurospora* which catalyses the reaction:

$$NH_2OH + DPNH + H^+ \rightarrow NH_3 + DPN + H_2O \qquad 13.$$

The reaction rate is increased two- to threefold by addition of FAD and is inhibited by the addition of cyanide. The E_0' of NH_2OH/NH_3 is +0.8 volts at pH 7. Extensive experiments with various substrates and inhibitors on an aldehyde oxidase preparation from liver have been carried out by Hurwitz (98). Model experiments with metalloflavoproteins and theories of metalloflavoprotein function have been presented by Mahler, Fairhurst & Mackler (99). McElroy & Green (100) and Totter & Cormier (101) have published additional papers on the properties of bacterial luciferase. This enzyme catalyzes a reaction involving FMNH, a higher fatty aldehyde, and oxygen with the emission of visible light. The enzymatic activities of the partially purified preparations are still rather complex, and it is not yet clear which properties are relevant. Rogers & McElroy (102) describe a dark mutant of *Achromobacter fischeri* in which luminescence is restored by the addition of a fatty aldehyde. In a second type of dark mutant the enzyme appears to be completely missing. Thus although the properties of luciferase indicate a possible role in normal electron transport the path must be a nonessential one.

Mammalian Cytochromes

Cytochrome-c.—The ion exchange methods of Margoliash have yielded cytochrome-*c* preparations containing 0.465 per cent iron corresponding to a molecular weight of 11,800. Such preparations have been found by Margoliash (103) to contain two histidine amino end groups and two internal histidine residues per molecule. One of the internal histidines has a hindered imidazole ring which does not react with fluorodinitrobenzene until the protein is treated with hydrogen peroxide which destroys the prosthetic group. This behavior is in accord with the views of Theorell & Akesson (104) that the heme iron is coordinated with a histidine residue. Experiments of Theorell had also shown that the cytochrome-*c* heme could be isolated from acid

hydrolysates of the protein as a thioether adduct with two molecules of cysteine (105).

The structure analysis of cytochrome-*c* was greatly advanced by Tuppy & Bodo (106) who subjected beef cytochrome-*c* to tryptic digestion and isolated a heme-containing peptide. After silver salt cleavage of the thioether links of the heme they found the amino acid sequence of the peptide to be as follows:

$$\text{Cys-Ala-Glu}\cdot\text{NH}_2\text{-Cys-His-Thr-Val-Glu-Lys} \qquad 14.$$

Two cysteine residues are present in the peptide. It should be noted that Paul's silver salt cleavage method (107) as applied by him to the intact cytochrome yielded an optically active hematoporphyrin. It is presumed that the heme carbon atoms linked to sulfur are optically active centers, and that the cleavage occurs with retention or inversion of configuration. After peptic digestion of the cytochrome Tuppy & Paleus (108) isolated a somewhat longer heme peptide:

$$\text{-Val-Glu-Lys-Cys-Ala-Glu}\cdot\text{NH}_2\text{-Cys-His-Thr-Val-Glu-} \qquad 15.$$

A heme peptide identical with the peptic product of beef cytochrome-*c* was also obtained from salmon cytochrome-*c*. On the other hand the corresponding peptide from chicken cytochrome-*c* contained serylglutamine instead of alanylglutamine between the two cysteine residues. The results of Sanger and co-workers (109) show that differences in the amino acid sequences of various mammalian insulins also seem to be localized in the segment between two linked cysteine residues.

Magnetic susceptibility measurements by Paleus, Ehrenberg & Tuppy (110) indicate that the heme peptide iron is coordinated by bonds of ionic character in acid solution and by bonds of covalent character in alkaline solution. In accord with this observation, Ehrenberg & Theorell (111) find that the sedimentation coefficient of the peptide increases as the solution is made more alkaline, suggesting the formation of a tetramer in alkaline solution. The bonding may occur through heme iron and histidine residues since the sedimentation coefficient drops in the presence of added histidine. Models of the peptide in α-helix configuration form a rigid strainless heme link through the two cysteine residues. The heme in the intact protein is pictured as surrounded by four parallel α-helix chain segments in square array. Bodo (112) reports the crytallization of the cytochrome-*c* obtained from the pectoral muscle of the king penguin.

Cytochrome-c_1.—The α-band of free cytochrome-*c* has a maximum at 550 mμ, whereas in heart mitochondria the corresponding maximum appears at 551.5 mμ. This discrepancy has been subjected to a painstaking analysis by Keilin & Hartree (113). When the particles were subjected to heat treatment the normal 550 mμ band position appeared, suggesting that a heat labile component which overlapped the cytochrome-*c* band had been destroyed. Exhaustive washing of the unheated particles, a treatment known to leach out most of the cytochrome-*c*, left residual particles with an absorption

maximum at 553 to 554 mμ. A cytochrome isolated in soluble form from the washed particles exhibited absorption maxima at 418, 524, and 554 mμ, was heat labile, nonautooxidizable, and spectroscopically inert to carbon monoxide and cyanide. The low temperature spectrum was identical with that previously assigned to a cytochrome-*e* by these workers (114). However the name cytochrome-*e* is now abandoned by Keilin & Hartree in favor of cytochrome-c_1 since the properties are identical with those of the pigment isolated from washed heart particles by Yakushiji & Okunuku (115) in 1941 and named cytochrome-c_1 by them. Cytochrome-c_1 in washed particles is reduced by succinate, and the reaction is inhibited by antimycin-A. The potential is stated to be between those of cytochromes-*b* and *c*, although the evidence presented for this degree of localization is not yet satisfactory. The control experiments on light scattering and the discussion of spectroscopy in this paper are of interest.

Microsomal cytochrome.—Mammalian liver contains a particulate heme protein spectroscopically distinct from the main mitochrondrial cytochromes and named cytochrome-*b'* by its discoverer, Yoshikawa (116), who observed a low standard potential and a cytochrome-*b* type of spectrum in particulate preparations. This cytochrome was localized in liver microsomes by C. F. Strittmatter & Ball (117) and termed cytochrome-*m* by them. Chance & G. H. Williams (82) referred to the same pigment as cytochrome-b_5 because of its spectroscopic resemblance to the cytochrome-b_5 observed by C. M. Williams (118) in homogenates of the midgut of *Cecropia* moth larvae.

P. Strittmatter & Velick (119) have isolated and purified this cytochrome from rabbit liver microsomes. The purified protein sediments with a single boundary and the molecular weight from sedimentation velocity and diffusion and from minimum molecular weights based upon total iron and total heme is about 17,000. The prosthetic group is removed quantitatively in acid acetone and is spectroscopically identical with the ferriprotoporphyrin obtained in the same manner from hemoglobin. In the reduced form the absorption maxima are at 423, 527, and 557 mμ. The pigment reacts with oxygen very slowly after enzymatic reduction but more rapidly after chemical reduction. In the latter case it is likely that autooxidation is mediated by reaction products or impurities in the reducing agents. No addition compounds with carbon monoxide or cyanide were detected spectrophotometrically. Velick & Strittmatter (120) have carried out stoichiometric oxidative and reductive titrations of the cytochrome and find that one oxidizing or one reducing equivalent are required for the interconversion of the fully oxidized and fully reduced forms. Oxidation reduction equilibria were established with indigo tetrasulfonate and with ferrous and ferric oxalate. Spectrophotometric equilibrium titrations with these reagents could be accounted for by a one electron change in the cytochrome. The potential, E_0', of the free cytochrome is +0.03 v. between pH 5.2 and 6.4.

The reduced pigment reacts very rapidly with ferricytochrome-*c* in a reaction that goes to completion in a one to one molecular ratio. Thus although

cytochrome-*c* is considered to have a shielded and fully coordinated heme iron there seems to be no serious barrier in the transfer of an electron from one protein to the other. The standard potential of the free cytochrome is more than 0.1 v. higher than values reported for the particulate form (116, 117). Although there is ample precedent for potential shifts arising from complex formation, the details of such effects are of the utmost importance in any interpretation of function.

*Cytochromes-*a *and* a_3.—In 1939 Keilin & Hartree (121) observed a series of absorption bands which were closely associated with those of cytochrome-*a* and which they termed cytochrome-a_3. This substance in crude suspension formed a carbon monoxide complex with a spectrum similar to the photodissociation action spectrum of the carbon monoxide-inhibited Atmungsferment of Warburg (122). Photodissociation of the cytochrome-a_3 carbon monoxide compound was observed by Chance (123), who scanned the difference spectrum of two carbon monoxide preparations while irradiating one of them at a wavelength outside the spectral region of interest. The difference spectrum corresponded approximately to the action spectrum of the terminal oxidase. Castor & Chance (124) have devised an ingenious optical system which in conjunction with a polarographic method for measuring oxygen consumption in solution enables them to make accurate determinations of photodissociation action spectra of the type originally obtained by Warburg. The results obtained by this method correspond to spectra of single heme proteins with no anomalous absorption bands. Cytochrome-a_3 is found to be the terminal oxidase in bakers yeast, mouse ascites tumor cells, and pig heart particle preparations.

Smith (125) has modified the cholate, trypsin, ammonium sulfate method of Smith & Stotz (126) for making a dispersion of cytochromes-*a* plus a_3. In such preparations the 605 mμ absorption band is attributable predominantly to cytochrome-*a* and the 445 mμ band chiefly to cytochrome-a_3. In agreement with the conclusions of Keilin & Hartree she observes that the two absorption bands change independently under various steady state conditions. Waino (127) describes the reduction of a similar cytochrome-*a* plus cytochrome-a_3 mixture with ferrocytochrome-*c* under anaerobic conditions. A good equilibrium constant was not obtained, but from the results he assigned a potential, E_0', of about +0.27 v. to the cytochrome oxidase. Since the calculations were based upon spectrophotometric observations made at 605 mμ the results, in view of the above findings, presumably apply to cytochrome-*a* and not to the oxidase.

Cytochromes in Microorganisms

Cytochrome-b_2 *and lactic dehydrogenase.*—The association of a characteristic hemeprotein spectrum, termed cytochrome-b_2, with the partially purified lactic dehydrogenase of yeast was originally observed by Bach, Dixon & Zerfas (128). The enzyme was crystallized by Appleby & Morton (129) in 1953 by methods the details of which have not yet appeared. This unusual

crystalline protein was found to contain FMN as a prosthetic group in addition to an equimolecular equivalent of ferriprotoporphyrin. With lactate as substrate the reduction of methylene blue and cytochrome-*c* were catalysed at equivalent rates, but ferricyanide was reduced about four times as rapidly. The microsomal cytrochrome of rabbit liver subsequently isolated by Strittmatter & Velick (119) proved to have a spectrum which in the region 300 to 600 mμ was nearly superimposable upon that for the yeast lactic dehydrogenase reported by Appleby & Morton. However the microsomal protein carried neither flavin nor lactic dehydrogenase activity. A much higher absorption by the yeast protein in the ultraviolet region suggests that it has a higher molecular weight than the liver cytochrome. The association of prosthetic groups on the yeast protein poses a number of problems of unique interest.

Boeri *et al.* (130) describe the preparation of an amorphous but highly active preparation of the yeast lactic dehydrogenase. The hematin iron content was 0.025 per cent, and the ratio of flavin to heme iron was one. The enzyme was specific for L(+)lactic acid. At pH 7.4 with $3\times10^{-7}M$ lactate and $5\times10^{-5}M$ cytochrome-*c* the turnover rate of the enzyme was 9000 per min. in a reaction that was first order with respect to cytochrome. The Michaelis constant for cytochrome-*c* was 1.85×10^{-5} and for lactate 3.5×10^{-4}. The enzyme was activated by versene.

Low potential cytochromes.—The organism, *Desulfovibrio desulfuricans*, contains a cytochrome which seems to be involved in sulfate reduction. Postgate (131) has now obtained this cytochrome in purified form from extracts of acetone-dried cells by chromatographic methods. The sedimentation constant, $S_{20,w}$, is 1.93×10^{-13}. Absorption maxima, reduced, occur at 419, 525, and 553 mμ, and the isoelectric point is 10.3 to 10.6. A remarkable feature of this protein is that it contains 0.92 per cent heme iron and two stably bound heme residues on a protein about the size of cytochrome-*c*. The standard potential, E_0', at pH 7.0, 30°C., is reported to be -0.205 ± 0.004 v. A potential of this low magnitude would be expected if the cytochrome were to be involved in sulfate reduction. Postgate considers that naming a cytochrome after the organism from which it is derived is cumbersome. This is certainly true in the case of *Desulfovibrio desulfuricans*, but in view of the unique properties of this protein his choice of the designation cytochrome-c_3 is somewhat misleading.

Newton & Kamen (132) report the isolation and partial purification of an acidic cytochrome from the photosynthetic obligate anaerobe, *Chromatium*. The protein is stable only between pH 6 and 8, and in its present state of purification contains both heme and nonheme iron. E_0' at pH 7 is reported to be -0.04 v. from equilibria with ferrous and ferric oxalate. The absorption maxima, reduced, are at 417, 520, and 552 mμ.

High potential bacterial cytochromes.—Kamen & Vernon (133) have surveyed a number of cytochromes in some photosynthetic and chemosynthetic organisms. In each case a soluble cytochrome was obtained and

carried through a few fractionation steps with variable degrees of purification. *Rhodospirillum rubrum*, *R. sphaeroides*, *R. capsulatis*, and *R. palustris* all yielded cytochromes with absorption maxima, reduced, at 416 to 418, 520 to 523, and 550 to 552 mμ and with potentials, E_0' at pH 7 of +0.31 to +0.33 v. Potentials were estimated spectrophotometrically from equilibria with ferro and ferricyanide. These cytochromes were reduced by mammalian cytochrome-*c* reductase preparations and by bacterial reductase preparations, but their reoxidation was not catalyzed by mammalian cytochrome oxidase preparations. The firmly bound prosthetic groups could be removed from the proteins by the silver salt cleavage method to yield products resembling the hematoporphyrin obtained in a similar way from mammalian cytochrome-*c*. In addition to the *c*-type cytochrome *Rhodospirillum* contains a carbon monoxide-binding pigment which Kamen & Vernon isolated in soluble and partially purified form. Since the prosthetic group was found to be ferriprotoporphyrin the substance was called a pseudohemoglobin. Chance & Smith (134) observed the same pigment in intact cells and considered it a terminal oxidase.

The cytochrome obtained from *Micrococcus denitrificans*, a nitrate reducer, exhibited absorption maxima, reduced, at 416, 522, and 550 mμ. The E_0' was estimated to be +0.25 v., and mammalian cytochrome oxidase catalyzed the reoxidation of the reduced form (133). Spectrophotometric studies of cytochrome steady states in intact cells of this organism were carried out by Sato and Chance [Chance (135)]. Reduction of oxygen and of nitrate is believed to occur through the same cytochrome sequence which is terminated by a carbon monoxide-binding pigment.

Smith (136) has studied cytochrome spectra in intact cells of *Bacillus subtilis* and *Micrococcus pyogenes* var. *albus*. The latter organism contains cytochrome-*a* but no cytochrome-a_3. The two pigments, therefore, do not invariably occur together. The ratio of *a* to a_3 in *B. subtilis* was different from that observed in heart muscle.

Metmyoglobin, Peroxidase, and Catalase

Metmyoglobin.—This protein reacts with hydrogen peroxide and alkyl peroxides to form a spectroscopically characteristic derivative which was originally supposed to be a dissociable peroxide complex. However investigations by George & Irvine (137, 138) revealed that the product was not a complex but a higher oxidation state of metmyoglobin obtainable not only with peroxides but with a variety of inorganic oxidizing agents. Since the product could be reduced back to metmyoglobin with one equivalent of ferrocyanide, it was formally equivalent to a myoglobin with the iron in the +4 valence state. George & Irvine (139) have continued their extensive investigations on this subject. An oxidative equilibrium was established with potassium chloroiridate and was formulated as follows:

$$Fe_p^+H_2O + IrCl_6^{2-} \leftrightarrow Fe_pO + IrCl_6^{3-} + 2\,H^+ \qquad 16.$$

Fe_pO is the ferryl form of the heme iron with a 4+ valence state. Participa-

tion of two hydrogen ions was deduced from the pH changes which occurred in reaction run in unbuffered solutions after taking account of the pKs of the protein. The formulation was consistent with the observed effects of pH, ionic strength, and temperature upon the apparent equilibirium constant. The results favor the ferryl structure for the higher valence state of metmyoglobin.

Magnetic susceptibility measurements on metmyoglobin and methemoglobin by the static method had indicated that the iron was coordinated by bonds of predominantly ionic character strictly comparable with those in hydrated ferric ions. Bennet *et al.* (140) have studied the magnetic properties of myoglobin and hemoglobin by the paramagnetic resonance method. The electronic state of the iron in metmyoglobin, methemoglobin, and hemin was found to be entirely different from that in the hydrated ferric ion in spite of the similar magnetic susceptibilities. From the similar behaviors of metmyoglobin and methemoglobin it was also concluded that there was no coupling between the magnetic moments of the four iron atoms in methemoglobin.

The changes in free energy, heat content, and entropy reported by George & Hanania (141) for the reactions of cyanide ion with metmyoglobin, and ferricytochrome-*c* are presented in Table I. These changes are in accord with the spectroscopic and magnetic evidence that the coordination positions on either side of the heme in cytochrome-*c* are occupied by nitrogenous groups, i.e., that the heme is in a crevice. The contrasting behavior of myoglobin is consistent with chemical evidence and with the crystallographic data of Kendrew & Parrish (142) that the heme of this protein is on the surface.

TABLE I

THERMODYNAMIC VALUES FOR THE FORMATION OF CYANIDE COMPLEXES OF FERRIMYOGLOBIN AND FERRICYTOCHROME-*c*

	G kcal M^{-1}	H kcal M^{-1}	S entropy units
Ferrimyoglobin	-11.4	-18.6	-24
Ferricytochrome-*c*	-8.3	$+1.1$	$+31.3$

Peroxidase and catalase.—An informative and somewhat surprising study of the reversible formation of cyanide complexes with ferro and ferri peroxidase has been presented by Keilin & Hartree (143). The ferri complex is more stable and is formed over a wider pH range. At pH 5.5 to 6.0 it is not reducible even by hydrosulfite, resembling free ferricatalase in this respect. Of particular interest was the observation that the ferroperoxidase cyanide compound undergoes photodissociation and that the quantum efficiency in the process is close to one. The only heme compounds hitherto known to undergo photodissociation were those with carbon monoxide.

It was found by Keilin & Mann (144) that peroxidase forms a number of spectroscopically identifiable compounds when treated with hydrogen peroxide. One of these, compound II, exhibited the kinetic properties of a rate-limiting intermediate in the peroxidase reaction formulated by Chance (145). The intermediate was unstable both in the presence and absence of acceptor, and early evidence that it exhibited measurable dissociation (146) was at the limit of experimental significance and was probably illusory. Further investigation revealed that the instability was attributable to the presence of impurities in the enzyme preparations and that a stable compound II could be prepared from properly purified enzyme. George (147, 148) working with a stable compound II found that it was not a peroxide complex, as previously supposed, but a higher valence state of the enzyme closely resembling the proposed ferryl ion form of myoglobin. Both compound II and compound I from which it is formed are produced by various inorganic oxidizing agents and contain one and two oxidizing equivalents respectively. Ferguson & Chance (149) have discussed aspects of the kinetics and stoichiometry of the formation of compound I by bromoiridate. They do not fully accept reaction mechanisms which fail to involve the formation of a hydrogen peroxide complex with the enzyme.

In a long paper characteristically rich in hemeprotein technology Keilin & Hartree (150) deal with coupled peroxidatic reactions catalyzed by catalase, peroxidase, and metmyoglobin. Manometric methods were used in comparing the coupled oxidations of different hydrogen donors by peroxide generated by the glucose oxidase system under standard conditions. A variety of specificities were observed which have a bearing on the functional differences between these proteins. The theory that catalase undergoes cyclic valency changes in peroxidatic reactions is discussed. The authors favor such a theory both for the peroxidatic and catalatic activities of catalase but present evidence only for the former since they consider the violent catalatic reaction too rapid for the observation of intermediate catalase compounds.

It has been found by Wang (151) that tetraethylenetetramine forms a ferric ion complex which has a high catalase activity. The second order rate constant, $d(H_2O_2)/dt = k_2$ (catalyst) (H_2O_2), is 1.2×10^3 sec$^{-1}M^{-1}$ compared to 5×10^6 for catalase and only 0.12 for hemoglobin. A detailed consideration of bonds lengths and bond angles in structural models suggests that both oxygen atoms in the peroxide coordinate with the iron and that the oxygen to oxygen bond becomes stretched, which lowers the activation energy for breaking the bond. Thus the O and the OH of the peroxide in an intramolecular reaction become two separate ligands on the iron and react with a second peroxide molecule to yield O_2 and regenerate the original complex. The model, like catalase, is inhibited by cyanide but, unlike catalase, it is activated by fluoride. It is suggested that an Fe-F bond is formed and that the peroxide displaces the fluoride more easily than than it displaces a hydroxyl group. Maehly (152) has studied spectrophotometrically the steps in the recombination of heme with the peroxidase protein.

SOME OXIDASES

Phenolase.—A true oxygen-transferring enzyme has finally been identified. The enzyme is the familiar phenolase which was found by Mason, Fowlks & Peterson (153) to utilize O^{18}-labeled molecular oxygen for the hydroxylation of phenols. The enzyme is known to contain two atoms of copper per molecule. The postulated reaction sequence is shown in Figure 1. One oxygen atom enters the phenol and the second one oxidizes the two cuprous ions to the cupric state and forms water. Regeneration of the reduced enzyme may occur by oxidation of the diphenol to a quinone or through reduction of the copper by some other reducing system. Hayaishi, Katagiri & Rothberg (154) have since found that the pyrocatechase of *Pseudomonas* transfers O^{18} from molecular oxygen to substrate in the oxidation of catechol to *cis-cis*-muconic acid. Absence of O^{18}-exchange with water makes the semialdehyde an unlikely intermediate. The positions of enzymatic oxidative

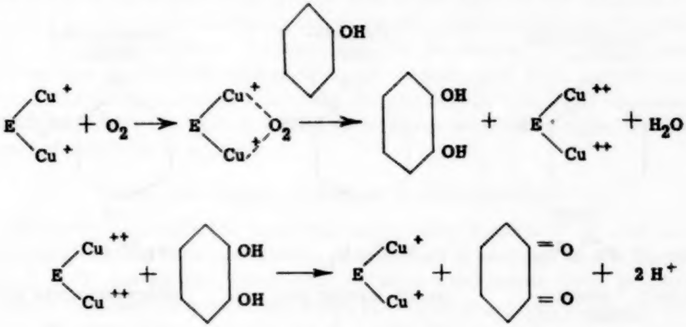


FIG. 1. The proposed mechanism of action of phenolase.

cleavage of three types of diphenol are shown in Figure 2. It is suggested that all three reactions involve transfer of molecular oxygen. When ring cleavage occurs it is possible that both atoms of molecular oxygen are transferred and a reducing system may not be required. The protocatechuic oxidase of *Pseudomonas fluorescens* was found by Stanier & Ingraham (155) to require oxygen, to be unaffected by high concentrations of catalase, and to show no trace of a quinone intermediate. For additional studies on pyrocatechase and protocatechuic oxidase, see Sistrom & Stanier (156) and MacDonald, Stanier & Ingraham (157).

Crandall (158) and Knox & Edwards (159) have purified homogentisicase from liver and have confirmed the participation of iron in the catalysis. Knox & Edwards (160) have identified the primary reaction product as maleylacetoacetic acid which is then isomerized to fumarylacetoacetic acid by an enzyme which has a specific requirement for glutathione.

Oxidases in liver microsomes.—A peculiar and unexplained type of TPNH requirement has appeared in studies of reactions of various types of

drugs catalyzed by liver microsomes. Thus Axelrod (161) reports that the oxidative deamination of amphetamine requires the simultaneous presence of TPNH and oxygen. Similar requirements were found by Mitoma & Udenfriend (162) in side chain oxidations and hydroxyiations of aromatic compounds. Recently Grant & Brownie (163) observed that a previous requirement for fumarate in the 11 β-hydroxylation of steroids catalyzed by a particulate fraction of adrenal cortex could be replaced by TPNH. The reviewer suggests that phenolase may provide the model for these reactions. If molecular oxygen and oxidizable metallo enzymes were involved the TPNH would serve to regenerate the reduced and active enzyme.

Uricase.—Mahler, Hubscher & Baum (164) have obtained a five thousand fold purification of uricase from pork liver mitochondria in a 10 to 30 per cent yield. In contrast with the results of previous workers this group has prepared a soluble enzyme which appears homogeneous by electrophoretic and sedimentation criteria and has a molecular weight estimated at

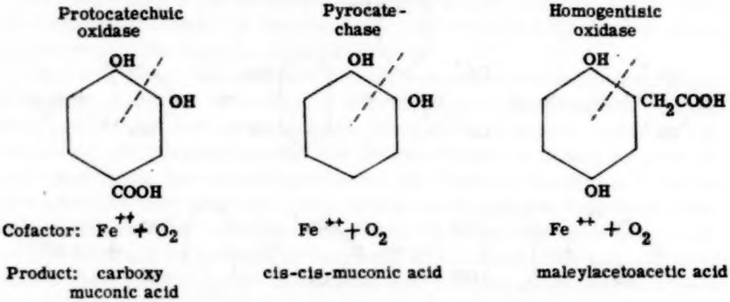


FIG. 2. The action of some diphenol oxidases.

100,000. The turnover number is 12,000 at the pH optimum, 9.0, and 38°C., Iron and zinc, formerly suspected as being active components of the enzyme, have been excluded, and the prosthetic group has been established as one atom of copper per molecule of protein. The enzyme is reversibly inhibited by cyanide. Diethyldithiocarbamate, by spectroscopic criteria, complexes with the copper without removing it from the enzyme, and causes activation instead of inhibition.

Canellakis, Tuttle & Cohen (165) have studied the action of crude uricase preparations using 2-C^{14} and 8-C^{14}-labeled uric acids as substrates. The reaction is complex and leads to urea and alantoin in phosphate buffers at pH 7.8 and 8.5, but in borate buffers at pH 7.2 the products are chiefly urea and alloxanic acid. In the latter case the intermediate has been tentatively identified as 5-ureido-2-imidazolidine-4,5-diol-4-carboxylic acid. Tests with O^{18} in the uricase reaction should prove to be of interest.

DPNH-oxidizing systems.—Three DPNH-oxidizing systems occur in *Streptococcus faecalis* [Dolin (166)]. An oxidase in crude extracts catalyzes

the reaction,

$$DPNH + H^+ + O_2 \rightarrow DPN + H_2O_2 \qquad 17.$$

and a DPNH-specific peroxidase utilizes the peroxide in catalyzing the reaction,

$$DPNH + H^+ + H_2O_2 \rightarrow DPN + 2\ H_2O \qquad 18.$$

Associated with the peroxidase is another enzyme which varies independently of the peroxidase and catalyses the oxidation of DPNH by dyes. Finally there is an enzyme which catalyzes the reaction,

$$DPNH + 2\ \text{Ferricytochrome-}c \rightarrow DPN + 2\ \text{Ferrocytochrome-}c + H^+ \qquad 19.$$

This is an interesting reaction since the organism contains no demonstrable cytochrome. Dolin has purified the peroxidase one hundredfold and finds it inactive with TPNH and with oxidizing agents other than hydrogen peroxide. The oxidase and the cytochrome-*c* reductase can be inactivated by removal of their prosthetic groups and reactivated by the addition of FAD. All of the DPNH-oxidizing activities were greatly diminished in cells grown on a flavin-deficient medium, but the proteins of the cytochrome reductase and the DPNH oxidase were still present since these activities in the extracts were increased thirtyfold by the addition of FAD. Enzyme yields were doubled in cells grown in an iron-free medium or under aerobic instead of anaerobic conditions.

Oxidative Phosphorylation in Mitochondria

Efficiency.—Methods for preparing active mitochondria from liver and the recognition that these contained a complete set of enzymes for the formation of ATP during the transfer of reducing equivalents from primary acceptors to molecular oxygen facilitated quantitative analysis of phosphorylation efficiency. Thus Copenhaver & Lardy (167) and Lehninger (168), found by direct methods that three molecules of ATP were formed from orthophosphate ions and ADP during the transfer of a pair of electrons from DPNH to oxygen. The observation of Lardy & Wellman (169) that addition of ADP is necessary for maximal rates of oxygen uptake by intact mitochondria has been utilized by Chance & Williams (170). These workers describe a vibrating platinum electrode which, with suitable recording equipment, may be used to measure the disappearance of dissolved oxygen in respiring mitochondrial suspensions. A slow oxygen consumption in the presence of substrate and in the absence of added ADP is accelerated four- to tenfold by the addition of ADP and then subsides when the ADP is depleted. Without correction for the basal rate, the molar ratios of ADP added, to the oxygen consumed during the period of accelerated respiration are 3.1, 2.6 and 1.8 when the substrates are respectively glutamate, β-hydroxybutyrate, and succinate. Since these figures are in agreement with the phosphorylation efficiencies for these substrates determined by direct methods, and since the results are the same regardless of the extent of acceleration over the basal level, it is assumed that the basal rate may be ignored.

The implication is that the basal rate, whatever its origin, is in some way shut off by the addition of ADP. The method is thus considered to give a measure both of the kinetics and the stoichiometry of oxidative phosphorylation.

Respiratory components in mitochondria.—There have been few available data on the concentrations of the various respiratory components of mitochondria. Estimates by optical methods of the relative concentrations in intact mitochondria have been made by Chance & Williams (171). For this purpose they used a split beam spectrophotometer to scan the optical density differences between reduced and oxidized suspensions of mitochondria. From these differences and with molar absorption coefficients that were reasonable approximations they estimated that cytochromes-a_3, a, c, c_1 (e in their terminology), and b are present in roughly equimolar concentrations. The amount of flavoprotein detected depends upon the method of reduction. It is, therefore, possible that different flavoproteins are segregated in different pathways. The total flavoprotein is estimated to be about four to five times the amount of any individual cytochrome, on a molar basis. The molar ratio of DPN to any individual cytochrome is from 10 to 40.

The idea that the cytochromes act in a sequence, $b \rightarrow c \rightarrow a \rightarrow a_3$, was advanced by Keilin (172, 173) from experiments with substrates and inhibitors and spectroscopic observations. This idea has been consistent with much subsequent work. The question as to whether cytochrome-b belongs in the sequence was taken up separately by Chance & Williams (174) since its participation in succinate oxidation had been denied (175). Phosphorylating mitochondria were employed and measurements were made of the rates with which the individual cytochrome absorption bands changed under various conditions. Such observations in a complex system do not establish a sequence but might be expected to reveal inconsistencies in the currently postulated sequence. None were found. It was concluded that cytochrome-b may be included in the sequence "with as much justification as the other components." However, previous conclusions based upon work with nonphosphorylating heart particles were still considered to be valid for those conditions. Rapid flow methods and a double beam spectrophotometer were employed in making the rate comparisons.

The sites of energetic coupling.—Evidence that at least one phosphorylation occurs in reactions between cytochrome-c and oxygen (176, 177) has been confirmed by Nielson & Lehninger (178) using as substrate ferrocytochrome-c that had been reduced with an inorganic catalyst and molecular hydrogen. These experiments were essential because previous work had left open the possibility that alternative pathways might have been utilized. Two phosphorylations were accounted for in a system in which reoxidation of cytochrome-c was blocked by cyanide [Borgstrom, Sudduth & Lehninger (179)].

The slowing down of mitochondrial respiration when the ADP becomes depleted looks like an approach to equilibrium. However when one considers that oxidation may occur independently of phosphorylation in uncoupled

systems, the phenomenon may be viewed as an inhibition. In the absence of a phosphate acceptor (ADP) something in the phosphorylating system accumulates and blocks electron transfer. Chance & Williams (180) have approached this problem by determining spectrophotometrically the fraction of each respiratory component that is reduced in five types of steady state that can be established by varying the concentrations of substrate, ADP, and oxygen. The basis of this approach is that sites of inhibition, and hence presumably of energetic coupling, can be detected by noting points in the respiratory sequence which mark a division between the accumulation of reduced and oxidized components in the chain. The model is provided by the behavior of antimycin-A. With this inhibitor cytochrome-*b* and components or the substrate side of cytochrome-*b* accumulate in the reduced form while cytochromes *c*, *a*, and a_3 becomes largely oxidized. The inhibition thus occurs between cytochromes-*b* and *c*, If the inhibitor could be removed cytochrome-*b* would be expected to become more oxidized and cytochrome-*c* more reduced. When two adjacent respiratory components change their reduction states in opposite directions in the above manner in a transition between steady states, a site of inhibition is localized on or between them. A search for the above type of crossover relationships in the transitions between the various steady states in phosphorylating mitochondria revealed possible inhibitions between DPNH and flavoprotein or cytochrome-*b*, between cytochrome-*b* and cytochrome-*c*, and between cytochromes-*c* and *a* (180). The results were thus consistent with previously established localizations.

Mechanisms of phosphorylation.—A characteristic of kinetic analysis is that it does not provide a unique interpretation in terms of molecular mechanism although it may serve to supplement other information and to exclude certain possibilities. In a complex case it is customary to treat the results by the simplest formulation compatible with all of the known facts, keeping in mind the fact that a wrong mechanism may still fit the kinetic data. Chance *et al.* (181) express their results and those of others in the scheme shown in Figure 3. It is assumed that the inhibition by ADP depletion occurs because one or more of the respiratory components accumulates in the form of an energy rich derivative which cannot transmit electrons or hydrogen unless a group acceptor is also provided. Unknown components X and I are pictured as participating in the phosphorylation. This is in accord with the idea of Lardy & Wellman (169) that several steps may be involved in the transfer of inorganic phosphate to ADP. This idea finds some support in recent experiments of Cohn & Drysdale (182), who, following the original work by Cohn (183), found that concomitant with electron transport in mitochondria there occurs an oxygen exchange between inorganic phosphate and water. The exchange is considerably more rapid in the phosphate going to ADP than it is in the inorganic phosphate pool. Moreover the results with ATP, but not with inorganic phosphate, were different depending upon whether the O^{18} used as a marker was originally in the phosphate or in the water. The suggestion was therefore made that part of the oxygen exchange

on the path to ADP occurred between phosphate and oxygen compounds other than water. The inhibited respiratory components in Figure 3 are pictured as DPNH and ferrocytochromes-*b* and *c*. Actually the data do not exclude any single respiratory component as a site of phosphorylation or energetic coupling nor do they establish one. That the respiratory components are inhibited in the reduced form is to some extent arbitary although arguments in support of the idea can be advanced.

The compatibility of Figure 3 with the experimental data was examined by subjecting it to a kinetic test. An abbreviated form of the scheme was used. Estimated concentrations of the components, rate constants or reasonable approximations, and appropriate functions were set in an analogue computer. The expected concentration changes and the crossover behavior of cytochromes-*c* and *a* were obtained. It should be noted that the known properties of the isolated respiratory components do not provide an easy molecular basis for the type of energetic coupling and inhibition postulated. The present work seems to define important properties of the phosphorylating system, but much chemical work on isolated systems appears to be necessary before any detailed mechanism can be accepted.

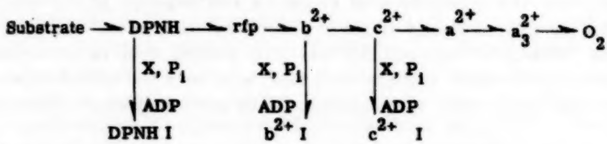


FIG. 3. Proposed respiratory sequence and sites of energetic coupling.

Properties of mitochondria.—Incubation of intact mitochondria in phosphate solutions leads to a loss both of oxidizing and phosphorylating activity. Hunter & Ford (184) have found that the bound DPN is split and released from the particles under these conditions. Activity could be restored by the addition of DPN in the presence of ATP and magnesium ions. Inactivation of mitochondria can be accelerated by the addition of hexokinase, glucose, and calcium or magnesium ions according to Ernster & Low (185). These workers restored respiration and phosphorylating activity by the addition of ATP in a reaction potentiated by manganese ions. The reactivation effects were most pronounced when succinate was used as substrate. Perhaps many of the inactivations of mitochondria will prove to be remediable.

The fragmentation of mitochondria with retention of oxidative phosphorylation activity has been reported by Cooper, Devlin & Lehninger (186). Cold digitonin solutions were used for this purpose. Fragments with an average particle weight of 50×10^6 were obtained by differential centrifugation. These were about 1/2000th the size of intact mitochondria. The P to O ratios were 2.6 with β-hydroxybutyrate as substrate and close to 1.0 when ferrocytochrome-*c* was the electron donor. Components of the fatty acid oxidizing system were eliminated and the adenosinetriphosphatase activity

was altered. It is hoped that this encouraging opening will lead to a chemical resolution of the complex functions of the particles.

Martius & Hess (187) have added to their original observations that oxidation and phosphorylation in mitochondria are uncoupled by thyroxine. Bain (188) and Mudd, Park & Lipmann (189) report that uncoupling by iodothyronine is antagonized by magnesium ions. Askonas and co-workers (190) have previously found that rather high concentrations of thyroxine inhibited phosphocreatine kinase by binding magnesium ions. A most striking development is the observation from Lehninger's laboratory (191) that the phosphorylating mitochondrial fragments are not uncoupled by thyroxine. Evidence has been obtained that the uncoupling by this hormone is related to osmotic effects in the intact mitochondria.

The dramatic inhibition of oxidation in mitochondria by minute amounts of antimycin-A has provided a useful tool in functional analysis of the respiratory chain where it appears to act by inhibiting reactions between cytochrome-*b* and cytochrome-*c*. The structure of this inhibitor has been studied by Tener *et al.* (192) and by Tener, van Tamelen & Strong (193). A complete structure which may account for all of the properties of this compound has been advanced by Tener (194) (see Fig. 4).

Photosynthesis

The photosynthetic process is currently considered to involve two phases: (*a*) the primary photochemical production of reducing equivalents and (*b*) their utilization for the reduction of carbon dioxide. The feasibility of this separation was established by Vishniac & Ochoa (195) in the demonstration that TPNH was produced photochemically in isolated chloroplasts. Although well characterized reactions were known in which carbon dioxide could be fixed and subsequently reduced by TPNH, these did not account for the apparent early production of 3-phosphoglyceric acid-1-C^{14} in photosynthetic experiments with $C^{14}O_2$ [Benson and co-workers (196)]. Evidence that the missing enzymatic step involved ribulose-1,5-diphosphate was provided by Weissbach, Smyrniotis & Horecker (197) and by Quayle *et al.* (198). After purifying the enzyme Weissbach & Horecker (199) found that the reaction, originally considered to be more complex, was actually,

$$\text{Ribulose-1,5-diphosphate} + CO_2 \rightarrow 2\ \text{Glyceric acid-3-phosphate} \qquad 20.$$

In accord with this reaction Wilson & Calvin (53) observed that ribulose diphosphate drops precipitously and glyceric acid-3-phosphate increases sharply in photosynthesizing cells when the light is turned off. Under such conditions the generation of ribulose diphosphate ceases, and reduced pyridine nucleotides are not produced to reduce the glyceric acid-3-phosphate to triose phosphate.

In their studies of quantum requirements in photosynthesis Warburg and co-workers had been using a beam of white light to compensate for respiration while making photosynthetic measurements with red or green light. Persistent variations in the results led Warburg, Krippahl & Schroder

(200) to the surprising discovery that catalytic amounts of bluegreen light greatly decreased the quantum requirement in photosynthesis. It was suggested that the effect was attributable to a light-induced transformation of an enzyme involved in photosynthesis. The catalytic effect of blue green light on quantum efficiency was utilized by Warburg, Krippahl & Schroder (201) in the determination of a photochemical action spectrum. A single-banded spectrum was obtained with a maximum near 460 mμ. In spite of superficial spectral similarities the participation of a flavoprotein was considered unlikely because such proteins had not hitherto exhibited rapidly reversible photochemical reactions. However, visual purple, a vitamin A aldehyde-protein complex [Ball, Goodwin & Morton (202)], has the properties which make it the preferred model for the reaction,

$$\text{Proferment} \underset{-\text{ bluegreen light}}{\overset{+\text{ bluegreen light}}{\longleftrightarrow}} \text{Luminoferment} \qquad 21.$$

Warburg and co-workers (203, 204, 205) have made a new attack on the primary photochemical process. *Chlorella* which has been respiring in the

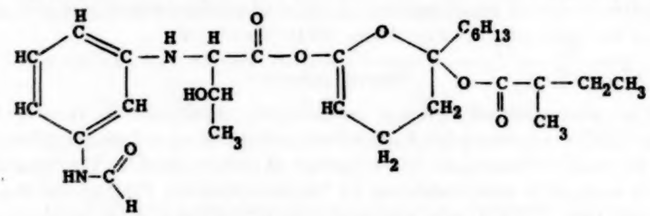


FIG. 4. Tentative structure of antimycin-A.

dark in the presence of carbon dioxide evolves one molecule of oxygen per molecule of chlorophyll when it is illuminated. The oxygen is not liberated in the dark at reduced oxygen pressure. Regeneration of the apparent chlorophyll-oxygen compound in the dark occurs only when respiration takes place in the presence of carbon dioxide. Work of Conant (206) provides some support for the proposed mechanism. The oxygen compound is considered not to be a molecular complex of oxygen and chlorophyll but to arise from the addition of water and carbon dioxide to the carbonyl side chain of the pigment in an endergonic reaction supported by oxidative metabolism. The oxygen liberated in the light arises from the combined water. After a rearrangement in the enlarged side chain the combined carbon dioxide is released photochemically at the reduction level of formaldehyde. The quantum efficiency of *Chlorella* is a function of its oxygen liberating capacity, and it is suggested that only the light absorbed by the chlorophyll-water-carbon dioxide adduct is photosynthetically active. Chlorophyll is thus made the prosthetic group of an enzyme which participates in intermediary compound formation in both the primary fixation and reduction process. Identification or detection of the liberated reduction product has not yet been reported. The system is a complex one and although the demonstrated carotenoid and

heavy metal functions are not yet clear their existence leads to no contradictions.

In the alternate theory carbon dioxide fixation is considered to occur primarily in the ribulose diphosphate cleavage to produce two molecules of 3-phosphoglyceric acid. Reduction would then require ATP and reduced pyridine nucleotide. Although, as stated above, TPNH has been shown to be produced photochemically the chief problem is to find a mechanism for its production. Calvin and his group have been trying to implicate thioctic acid as a primary electron acceptor since this would permit entry of electrons in the metabolic cycle at a potential sufficiently low to permit reduction of pyridine nucleotides. However attempts to establish experimentally the direct participation of thioctic acid in photosynthesis have been equivocal (207, 208, 209).

The synthesis of glucose in photosynthetic organisms requires the production both of reducing equivalents and ATP. Whatley, Allen & Arnon (210) have reported that under anaerobic conditions in the absence of carbon dioxide, chloroplasts supplemented with FMN, magnesium ions, and ascorbic acid, catalyse the photosynthetic phosphorylation of ADP to ATP. The reaction is stimulated by menadione, phthiocol, and vitamin K [Arnon, Whatley & Allen (211)], and no oxygen is involved in the process. Although the presence of ascorbic acid is a possible source of complication, the authors postulate that the photoelectrons generate ATP on their path back to the oxidizing equivalent produced in the primary photochemical reaction. Fraenkel (212) has reported photosynthetic phosphorylation in extracts of *Rhodospirillum rubrum*.

When carbon dioxide is present in photosynthesizing chloroplasts, Allen *et al.* 213) find that photosynthesis occurs with the evolution of oxygen. The molar ratio of oxygen evolved, measured manometrically, to carbon dioxide absorbed, measured with C^{14}, was unity. It is assumed that under these conditions the carbon dioxide acts as the ultimate electron acceptor and that the photochemically produced oxidizing equivalent is evolved as oxygen.

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THE CHEMISTRY OF PROTEINS AND PEPTIDES[1,2]

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This review will follow the now well established pattern of starting out with apologies to the authors of the many papers which have been omitted from consideration. The limits of time and space and of human endurance and enthusiasm are responsible that this is no more than a random sampling of some of the tidbits of scientific progress in the field of the organic chemistry of peptides and proteins over the past two years. Within this limited field, many papers were excluded from consideration because they were either accidentally not seen by the reviewer or not readily accessible to him or incomprehensible to him on linguistic or mathematical grounds. And this surely included some of the best.

Also not included, in the hope that they would be covered elsewhere in this issue, was most of the interesting work on the hemoglobins, on the activation of the zymogens, as well as many other aspects of the chemistry of enzymes, virus, etc.

Another topic which was regretfully omitted is the chemistry of the amino acids. But it appears probable that the many amino acids newly discovered in plants in recent years are of metabolic importance rather than of protein-structural significance.

The dividing line between peptides and proteins has just about disappeared in recent years unless one includes insulin with the peptides and arbitrarily proclaims as proteins only peptide chains containing more than one hundred amino acid residues. Proteins and peptides will, therefore, be dealt with jointly and often used as synonyms.

Isolation and Purity of Proteins

General methods.—Recent years have witnessed several isolated instances where the application of new and more refined fractionation methods has revealed heterogeneity in presumably pure proteins and has permitted separation of the components. This heterogeneity is not of the type discussed by Colvin *et al.* and so aptly reviewed by Ogston last year (1). A given batch of pooled serum albumin may well represent a mixture, with Gaussian distribution, of many identical molecules with a decreasing number of molecules differing in one, two, or even ten amino acids from the "standard."

The fact that serum albumin from individual pathological cases differs from the norm in its optical rotation (2) has no such philosophical implica-

[1] The survey of the literature pertaining to this review was completed in November, 1955.

[2] The following abbreviations are used in this chapter: ACTH for adrenocorticotropin; DNP for dinitrophenyl group; FDNB for fluorodinitrobenzene; TMV for tobacco mosaic virus.

tions but suggests caution in the interpretation of results obtained with pooled, or with single individual samples of proteins, particularly in work of great accuracy. Fortunately most protein analytical methods do not belong in this category. The marked differences in individual pathological globulins will be discussed later. Another interesting finding is that the milk of individual cows contains definitely differing ratios of the two components of β-lactoglobulin, with some samples containing only one of these components (3). Such differences appear to be genetically determined.

Differences in the amino acid composition of strains of virus and of biologically identical hormones and enzymes from different species have been known for a number of years. This year has brought the first structural localization of these differences. It appears of possible significance that sheep and pig insulin were reported to contain small and variable amounts of a peptide sequence which is characteristic of cattle insulin (4); contamination in the slaughter house, however, was not excluded. Another possible source of heterogeneity stems from secondary chemical changes occurring during isolation or storage. It appears probable that the two components of certain insulin and lysozyme preparations (5, 6), as well as those present in glucagon, are attributable to such causes.

Commendable attempts to apply as many of the more stringent fractionation techniques to as many proteins as possible are continuing. Thus Condliffe used partition chromatography for the isolation of micro-amounts of pure oxytocin and vasopressin (7). Andersen (8) and Pierce (9) have fractionated the A and B chains derived from oxidized insulin by means of partition chromatography and countercurrent distribution, respectively. The latter technique was used by Craenhals & Leonis (10) with lysozyme after a suitable solvent system had been selected. Starch electrophoresis was used to confirm the presence of two components in ribonuclease [Raacke & Li (11)]. Boardman & Partridge (12) found the weak cation exchange resin IRC-50, XE 64, adaptable to the separation of the hemoglobins. Previously only basic proteins had been successfully separated on such columns. Porter (13) and Hirs (14) used partition and XE 64 chromatography, respectively, for the study and separation of chymotrypsinogen and α-chymotrypsin. A strong cation exchanger (Dowex 50) was used successfully by Boman in the separation of phosphatases with ionic strength gradient elution at pH 7.2 (15). More recently the same author fractionated the serum proteins with Dowex 2, an anion exchange resin (16).

A new type of ion exchanger particularly suited for macromolecular materials was introduced in recent years by Peterson & Sober (17). These authors described the preparation of cellulose derivatives carrying weakly or strongly acidic or basic groups. With the anion exchanger DEAE-SF (diethylamino ethyl groups on wood cellulose) remarkable fractionations of serum became possible (18) while a basic cellulose derivative, triethanolamine proved of great use in the fractionation of nucleic acids.

Purification of specific proteins and peptides.—The purification of several hormones has approached the ever retreating goal of homogeneity a little closer during this year. The exciting work of tracking down the "anti-insulin" in insulin preparations has reached its goal in the crystallization of the new pancreatic protein hormone, glucagon, by Staub *et al.* (19). This is probably the first protein to be crystallized from urea solution (1 *M*), but it must be noted that its probable molecular weight of 4200 places it into the growing group of natural products which are too big for peptides and too small for proteins. The absence of cystine from glucagon explains its resistance to alkali, the property first used in showing that the hyperglycemic action of many insulin preparations was attributable to a separate chemical entity. Glucagon also is free of proline and isoleucine but, in contrast to insulin, contains both tryptophan and methionine.

Pure melanophore-stimulating peptides of high activity have been isolated by three research groups. Unfortunately they have almost nothing in common. The preparation of Porath and co-workers (20) was homogeneous in a zone electrophoretic setup, which has yet to be described, and isoelectric about pH 5; that of Lerner & Lee (21) was homogeneous during paper electrophoresis over a wide range of pH, as well as in countercurrent distribution (100 transfers). Its isoelectric point was between pH 10.5 and 11. Porath's suggestion that the latter preparation might be a complex between a basic inert material and the hormone appears improbable in view of the fact that it contained no histidine while Porath's material contained this amino acid. The opposite relationship is more likely. Finally, another preparation was described by Benfey & Purvis (22) which was both purified and tested for homogeneity by countercurrent distribution. Its composition resembled that of Porath's preparation, but it lacked valine and alanine. The active principle appears to belong to the 4000 molecular weight class.

Another active peptide which is being isolated in various form by various workers is hypertensin. The preparation described by Skeggs and co-workers (23, 24) appeared homogeneous in countercurrent distribution and starch electrophoresis (pH 4.2 and 9.3). It was isoelectric at pH 7.7 and its composition was (Asp, Pro, Val, Ileu, Leu, Tyr, Phe, Arg, His_2),[3] lacking several amino acids which were found by Kuether & Haney (25) in their unusually active but unstable preparation of unproven purity. In an earlier study, aspartic acid and leucine (or isoleucine) had been found as N- and C-terminal amino acids in yet another preparation, though by qualitative analysis only [Bumpus & Page (26)], but the N-terminal amino acid was not found by Kuether in his preparation.

[3] The customary, self-evident abbreviations for amino acid residues are used. A dot after the three-letter symbol (for R) denotes an R—CO— group, and a dot before the symbol the —NH—R group. Commas between symbols denote an unknown amino acid sequence. In contrast to others, parentheses will be used only to indicate uncertainty and will not be used for the amide group. thus glutaminyl = $.GluNH_2$.

The thyrotropic hormone of the pituitary has also been further purified. Its biological purity is more firmly established than its physicochemical homogeneity, although ultracentrifugal data (its sedimentation constant is 1.0) and final chromatographic fractionation suggests a single active molecular species [Fels, Simpson & Evans (27)].

The isolation of homogeneous fractions carrying ACTH activity has been overshadowed by their structural analysis. One aspect that remains unexplained is the occurrence in hog pituitary extracts of seven active components which were laboriously separated by countercurrent distribution by one group of workers (28), while the other workers in the field appeared content with the homogeneity of fractions which comprised, presumably, the bulk of the ACTH activity of their extracts (29, 30, 31). These may again be instances of families of closely related active principles, possibly in part produced in the course of isolation (cf. lysozyme, insulin, glucagon). Sheep corticotropin was isolated by dioxane fractionation followed by zone electrophoresis, chromatography on IRC-50, and finally countercurrent distribution (31).

Adenosinetriphosphate-creatine transphosphorylase is an enzyme from muscle which has been obtained in crystalline and probably pure state by Kuby, Noda & Lardy. Isolation was largely based on alcohol fractionation in the presence of divalent metals. The enzyme was well characterized by physicochemical methods (32, 33).

Cytochrome-*c* has for the first time been obtained in crystalline form, oddly enough from the king penguin [Bodo (34)]. A number of myoglobin preparations from different species have also been crystallized, and several of them have been crystallographically compared (35).

Simplified procedures were described for the isolation of pure pituitary growth and lactogenic hormones and for taka-amylase (36, 37, 38).

From thermophilic bacteria, grown at either 35° or 55°C., a crystalline and homogeneous α-amylase has been isolated by Campbell (39) by classical protein chemical techniques. This enzyme has the remarkable property of losing only 10 per cent of its activity in 1 hr. at 90°C. if prepared from the hot cultured bacteria, but becomes over 90 per cent inactivated when derived from the 35°C. culture. Such a system should be of greatest value in our search for an understanding of protein structure in general.

Another unusual case history in enzyme and protein chemistry has been reported by Smith and co-workers (40). These authors isolated a crystalline mercury salt of a protein as a by-product of the crystallization of papain (41). The protein had no proteolytic activity. However, its high isoelectric point (10.5) and its composition (high arginine and tryptophan, low histidine and glutamic acid) resembled that of lysozyme, and upon assay it proved to have one-fourth of the lytic activity of egg white lysozyme. It was not an —SH protein but seemed to bind three atoms of mercury in a complex involving its three histidine residues and three sulfate ions.

"Crystalline and homogeneous" proteins of a few years ago are now often fruitful sources for the isolation of new pure proteins. The finding of glucagon in insulin has been discussed. The properties of a new proteolytic enzyme isolated from commercial crystalline trypsin have not yet been described in detail [Weil (42)]. On the other hand, the separation of the toxic and lecithinase activities of the venom from *Crotalus terrificus*, and of crystalline crotoxin by IRC-50 chromatography at pH 6.87 has been clearly demonstrated (43, 44). The fact that this seemingly homogeneous protein represents a firm complex of two chemically distinct proteins, one acidic and one basic, has been demonstrated also by Fraenkel-Conrat & Singer who isolated from it two DNP derivatives of different amino acid composition (45).

Certain types of research seem to border on sacrilege, in the personal opinion of this reviewer. The isolation of strepogenin from partial hydrolysates of insulin belongs in that category. Be that as it may, progress is being made in that an active pure pentapeptide (Ser.His.Leu.Val.Glu) has been isolated (46), even though it represents only 2 per cent of the total activity of the digests; crude peptide fractions had previously been isolated which were 10 times as active. It has been known for some time that a considerable number of peptides show this growth stimulating activity towards microorganisms, but its nonspecificity appears to know almost no bounds. Thus several synthetic peptides on the path to oxytocin (see later) were also found active, and no single amino acid or sequence of amino acids seems to be required for the effect [Woolley *et al.* (47)].

Considerable attention has been paid during the past year to the nucleoproteins from calf thymus, and particularly the histones which can be isolated from them. Fractionation on IRC-50 is not here the ideal method, since the more basic half of the protein remains bound to the resin. However, this method has yielded what appears to be a small (10 per cent) homogeneous fraction characterized by the absence of histidine, methionine, cysteine, and tryptophan, and an extraordinarily high content in lysine and alanine [Crampton *et al.* (48)]. A fraction of similar composition, and apparently of low molecular weight (11,000 to 16,000), has also been obtained by Davison *et al.* (49, 50) and by Bakay *et al.* (51) by entirely different methods, in better yield though possibly less pure. Conversely, a fraction of high molecular weight (about 130,000) was obtained by the same groups of workers; this fraction contained tryptophan, cysteine, methionine, histidine, and much arginine. The work of Lucy & Butler (52) further indicates that these two main histone fractions are associated with deoxyribonucleic acid fractions of differing composition.

Porter (53) has continued his attempts to fractionate rabbit γ-globulin and specific antibodies by partition chromatography. This family of proteins previously characterized as identical in composition, in N-terminal pentapeptide sequence, and in physiocochemical parameters, proved heterogeneous

but impossible to resolve by this new technique. With an immune globulin, some inert γ-globulin could be separated from the antibody, but not vice versa. Two different antibodies against ovalbumin were actually detected at an early stage as compared to later in the course of immunization.

The purification of human fibrinogen by ether fractionation was reinvestigated by Kekwick (54). The final product was free of other components of the clotting mechanism and was 97 per cent clottable.

Structural Analysis of Proteins

Amino acid analysis.—Amino acid analysis, like elementary analysis for simpler compounds, is a necesssary evil. The research men who develop methods which can be used by others merit the gratitude of the profession.

Moore & Stein (55) have improved their ion exchange system for the analysis of amino acid mixtures, notwithstanding the fact that their 1951 model was still leading the field. A single column of Dowex-50-X4 now suffices for the separation of all common amino acids and many other natural amino compounds. It is only to be hoped that the advocated resin, or its equivalent, will remain readily available. Elution by a buffer gradient gives improved separation, and a modified ninhydrin reagent gives better analytical evaluation (56).

A simple technique for amino acid analysis was described by Levy (57, 58) in which the amino acids were dinitrophenylated and determined colorimetrically after separation by two-dimensional paper chromatography. The method has been reinvestigated and slightly simplified by obviating the use of a pH-stat (45). It is almost certainly less precise than the ion exchange method because of appreciable correction factors for losses incurred during formation and separation of the DNP derivatives of the amino acids, but it is very much easier and cheaper to set up and will give more data in a shorter time. The DNP method is eminently suitable for characterization of the composition of peptides, where only stoichiometrically related components are of interest, and traces of other amino acids are only regarded as evidence of contamination and not quantitatively evaluated.

A variation of this theme, transformation of the amino acids to the colorless phenylthiohydantoins and their column separation, has been suggested by Sjøquist, but details have not as yet been published (59). Unless the yields are quantitative, no advantage of this over the DNP method is apparent.

The possibility of using the ninhydrin reaction after paper chromatographic separation as a quantitative method for amino acid analysis continues to be explored. Some authors have obtained satisfactory results (60, 61), while others come to completely negative conclusions (62). Big correction factors are certainly necessary, as a result of losses of amino acids during chromatography. Isherwood & Cruickshank (63) have suggested

dinitrophenylation for the detection of the amino acids on two-dimensional chromatograms, and for the quantitative evaluation of the spots.

Table I contains a list of a few of the many proteins and peptides analyzed during the past two years by current analytical procedures.

TABLE I

RECENT ANALYSES OF AMINO ACID COMPOSITION OF PROTEINS AND PEPTIDES

Proteins	Analytical Method	Reference
ACTH	DNP-method	(64)
Antibodies (rabbit)	Ion exchange	(65)
Amylase (saliva)	Ion exchange	(66)
(Taka-)	Starch chrom., chem.	(67)
Carboxypeptidase	Ion exchange	(68)
Collagen, Procollagen	Ion exchange	(69)
Crotoxin (2 components)	DNP-method	(45)
Elastin	Ion exchange	(70)
Feathers, Down, etc.	Ion exchange	(71)
Hemoglobins (A, B, C, F)	Ion exchange	(72, 73)
Hypertensin	Ion exchange	(24)
Insulin, B-chain	DNP-method	(57)
α-Lactalbumin	Ion exchange	(74)
Myeloma globulins	Ion exchange	(75)
Myoglobin	Ion exchange	(76)
Papain	Ion exchange	(77)
Silk Fibroin (*Bombyx mori* and Tussah)	Ion exchange	(78)
Ribonuclease	Ion exchange	(79)
Tomato Bushy Stunt Virus (Strains)	Ion exchange	(80)
Wool	Ion exchange	(81)
X-Protein (from TMV)	Ion exchange	(82)

Methods for N-terminal residues and sequences.—In the study of N-terminal residues the FDNB method continues to be generally preferred. The reaction conditions employed by most workers are similar to those proposed by Sanger in 1945, though the reaction is also often performed in aqueous solution. The isolation and identification of the DNP amino acids is now generally based on paper chromatographic procedures although buffered celite columns are also used. Perplexing results are occasionally obtained. Thus Levy & Li (83) found that the N-terminal serine of ACTH could be recovered in only 10 to 20 per cent yield, although considerable evidence indicated the stoichiometry of the reaction of FDNB with the expected four lysine and one serine amino groups of this peptide. This study also contains interesting comparative data on the kinetics of the reactions with FDNB,

phenylisothiocyanate, and carbon disulfide. Low yields of DNP-amino acids (30 to 50 per cent), not duplicated in control mixtures, have also been reported by others (84); a particular lability of peptide-linked DNP-residues appears to constitute the only explanation of these discrepancies.

Splitting of peptide bonds is also occasionally observed upon prolonged treatment with FDNB (85). Earlier work on carboxypeptidase by Thompson indicated a particular lability of an N-terminal asparaginyl-serine bond, and qualitative data on the old yellow enzyme, recently republished, suggest that N-terminal aspartic acid can be split off also from adjacent leucine or valine residues (86). In view of the proven stability of other DNP-aspartyl-bonds in the serum albumins and γ-globulins, one is inclined to suspect that the labile end group in the yellow enzyme is asparagine rather than aspartic acid. Weygand suggests a β-peptide linkage as a possible explanation (86).

In consequence of the increasing importance and use of DNP derivatives of all types of amines, several studies have concerned themselves with methods for their separation and identification and description of their spectra, rotation, etc. (58, 87, 88, 89). Simplified procedures for the preparation of DNP-amino acids (90), and of α-DNP-histidine in particular (91), have been described. The transfer of the DNP group from phenolic to amino groups has been observed to occur in hot bicarbonate solution (92). Even more surprising is Middlebrook's observation that di-DNP-tyrosine loses the O-DNP residue upon prolonged washing with water (93).

Jutisz & Ritschard (94) have adapted the Holley reaction scheme for stepwise degradation to DNP-peptides, but it remains to be seen whether application of this method to natural unknown peptides will be feasible.

For stepwise degradation from the amino end, the phenylisothiocyanate method alone has been applied to natural peptides, although various related and unrelated methods have been suggested. Two modifications in the procedure of the Edman degradation have been developed in the reviewer's laboratory for smaller peptides and proteins, respectively. Peptide thiocarbamates were subjected to the acid-catalyzed cyclization reaction in very dilute solution, the reaction being followed spectrophotometrically. When the characteristic absorption band at or near 265 mμ reached a maximum, the phenylthiohydantoins were extracted with ethyl acetate, the aqueous solution dried in vacuo and again subjected to phenylisothiocyanate, followed by the cyclization reactions (95). In this manner it may be possible to degrade peptides of serine, threonine, and cystine without appreciable decomposition of the phenylthiohydantoins. For proteins, small strips of filter paper were advocated as carriers throughout the procedure, thus ensuring a great reacting surface for the acid-insoluble phenylthiocarbamyl proteins (96). The preparation of the thiohydantoins of serine, threonine, and cystine was described by Levy, as well as a technique for regeneration of the amino acids from thiohydantoins by acid hydrolysis (97, 98). Iodophenylisothiocyanate has also been proposed as an alternate reagent (99).

Leucine aminopeptidase has been advocated by Smith and co-workers as a tool for structural analysis from the amino end, and this enzyme appears to show much promise (100, 101, 102). Its more extensive use has probably been limited by its unavailability. Through the use of this enzyme White could confirm the N-terminal sequence of ACTH and demonstrate that the integrity of this part of the molecule was essential for hormonal activity (103).

Methods for C-terminal residues and sequences.—Various chemical methods for analysis of, or from, the C-terminal end have been investigated intensively. Hydrazinolysis, with isolation of the C-terminal amino acid as DNP derivatives (104), has been critically studied and found to be seemingly reliable for all C-terminal amino acids except cystine and tryptophan [Niu & Fraenkel-Conrat (105)]. The dicarboxylic amino acids are recovered only in low yield and their mono-amides not at all under the conditions employed. Arginine yielded ornithine in poor yield. C-terminal peptides have been obtained in several instances. Ammonolysis has been investigated as an alternate technique but appears to require higher temperatures and shows no advantages compared to hydrazinolysis [Chambers & Carpenter (106)].

Reduction, by means of $LiAlH_4$ or $LiBH_4$, of the C-terminal amino acids to the amino alcohol appears to have been abandoned by the original sponsors. However, the latter reagent has been reinvestigated quite intensively by Grassmann and co-workers (107, 108, 109). Those authors conclude that no untoward reactions such as reduction of amide or peptide bonds occurs, even in several hours in boiling tetrahydrofuran; in contrast the results of Crawhall & Elliott indicate appreciable reduction of the amide bonds (110, 111).

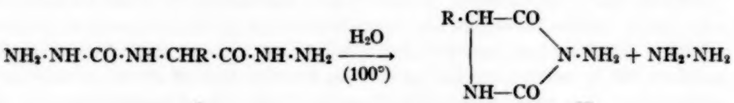
According to Grassmann, reduction of esters is close to quantitative. Without prior esterification, most peptides are quite resistant to reduction, but the C-terminal alanine of insulin is reduced by up to 20 per cent (108). The reduction products, the amino alcohols, are isolated and determined as DNP-derivatives (89, 108). It is to be hoped that this method can be applied with equal success by others and that its results can be confirmed by other methods. Thus, a comparative study led Bailey to conclude that lithium borohydride is much superior to lithium aluminum hydride or aluminum hydride and that even under reflux "there is little risk of amide reduction"; the latter statement is supported by only one experiment with a 3 hr. reduction period (112).

Bailey has further developed a scheme which may extend the scope of the reduction method to permit stepwise degradation from the carboxyl end (113). The principle of this method is that the reduction product, the N-acyl amino alcohol, is caused to undergo acyl shift to the O-acyl amino alcohol. This ester may again be reduced by $LiBH_4$ to the free amino alcohol derived from the C-terminal amino acid, and the N-acyl amino alcohol corresponding to the penultimate amino acid. This method must yet await the test of consecutive execution with a natural peptide.

Attempts to make use of the thiohydantoin reaction (Schlack and Kumpf) are continuing. While the conclusions of several authors were pessimistic [summarized by Jutisz (114)], some confirmatory results were obtained with ovomucoid, insulin (115), and lysozyme (116). In the case of ovalbumin, alanine is split off (115), although this is no longer regarded as a C-terminal residue (105, 47). Several amino acids cannot be detected by this procedure (115). In the case of bovine serum albumin, alanine has been independently identified in three laboratories as the only C-terminal amino acid, use being made of hydrazinolysis, the thiohydantoin method, and carboxypeptidase, respectively (105, 116, 118). A paper chromatographic method for the separation and identification of the thiohydantoins formed from the C-terminal amino acids has been described (116), as well as the application of the paper strip carrier technique (96) to the problem (119).

In continuation of a series of publications describing the application of various end-group methods in "subtractive" manner, Fox *et al.* have now applied this principle to the Schlack and Kumpf reaction (120). In view of the difficulties in obtaining the thiohydantoins in good yields for positive identification, the subtractive approach may well be of value in this instance. A similar case is that of the N-terminal DNP serine of ACTH which is more definitely proven by the subtractive method than by direct analysis (83). The Dakin-West reaction was also proposed, in subtractive manner, for C-terminal analysis (121). However, in general, positive identification and determination appears preferable, and the reliability of subtractive techniques will always be limited by the accuracy of amino acid analytical methods. It appears doubtful that either the advocated changes (120) in end-group terminology or in methodology will prove generally acceptable.

An ingenious method for simultaneous analysis of both ends of carbobenzoxypeptides has been proposed by Schlögl and co-workers (122). When such a peptide is hydrazinolyzed, the N-terminal amino acid forms a car-



I II

bamido-dihydrazide (I) which in boiling water cyclizes to an aminohydantoin (II); the latter can be extracted from acid solution with ether and then hydrolyzed for identification of the N-terminal amino acid. The determination, in the aqueous fraction of the free, originally C-terminal amino acid, is performed as customary after hydrazinolysis.

The results of many isolated end-group and sequence analyses are summarized in Table II. Proteins which were studied in more detail will be discussed below.

TABLE II

Terminal Amino Acids and Sequences of Various Proteins*

Protein	N-terminus	C-terminus	Reference
Actin	none	.His.Ileu.Phe	(123)
ACTH	Ser.Tyr.Ser.Met.Glu.	(.Pro).Leu.Glu.Phe	(30, 103, 124, 125, 126)
Chymotrypsinogen	Cy(S-).	none	(127)
Chymotrypsin-π	Cy(S-).,Ileu.Val. (2 chains)	.Arg	(127–130)
Chymotrypsin-δ	Cy(S-).,Ileu.Val. (2 chains)	.Leu	(127–130)
Crotoxin (sol. DNP-)	Ser.	.(Ser,Phe).Thr.Asp	(45)
Cytochrome-*c*	His., His. (2 chains)	—	(131)
Glucagon	His.	.Thr	(19, 132)
Growth hormone	Phe., Ala. (2 chains)	.Phe, .Phe (2 chains)	(133, 134)
Myosin	none	(.(I)leu)†	(123)
Papain	Ileu.Pro.Glu.		(135)
Pepsin	Leu.Gly.Asp.Asp.His.Glu.	.Val.(I)leu.Ala	(136, 137, 138)
Prolactin	Thr.Pro.Val.		(139)
Ribonuclease	Lys.Glu(NH_2).Thr.Ala.	.Ser.Val	(105, 140)
Taka-amylase	Ala.	.Ala,.Ser,.Gly (3 chains)	(104, 141)
Tobacco mosaic virus (Common and several strains)	none‡	.Thr.Ser.Gly.Pro.Ala.Thr	(142–145)
Tobacco mosaic virus (H.R. Strain)	none	.Thr.(Thr, Ala).Pro.Ala.Thr	(142–145)
Triosephosphate dehydrogenase	Val.,Val. (2 chains)	.Met,.Met (2 chains)	(146, 147)
Tropomyosin	none	.Ala.Ileu.Met.Thr.Ser.Ileu	(123)
Trypsin inhibitor (soybean)	Asp(NH_2).	(.Leu)	(105, 148)
Trypsinogen	Val.Asp.Asp.Asp.Asp.Lys.Ileu.Val.Gly.	none	(84, 149, 150, 151)
Yellow enzyme (old)	(Asp(NH_2).Leu,Asp(NH_2).Val., Glu.)		(86)
Collagen	none	.Gly,.Ala (2 chains)	(69, 107, 108, 109, 152, 153)
Fibrin clot (bovine)	Tyr.,Tyr.,Gly.,Gly.,Gly. (5 chains)		(93)
Gliadin (wheat)	Phe.,His. (2 chains)		(154, 155)
Gliadin (rye)	Phe.,Glu. (2 chains)		(154, 155)
γ-Globulin (rabbit)§ (and antibodies)	Ala.Leu.Val.Asp.Glu.		(156, 157)
Glycoprotein (acid, plasma)	none		(158)
β-Lactoglobulin	Leu.,Leu.(Leu.Val.) (2 chains)	.Ileu,.Ileu (2 chains)	(96, 105)
Myoglobin	Gly.Leu.(Ser.)		(96, 159)
Ovalbumin	none	.Val.Ser.Pro	(105)
Procollagen	Asp.,Ala.† (2 chains)	.Gly,.Val,.Ala (3 chains)	(69, 107, 108, 109, 152, 153)
Salmine	Pro.Arg.Arg.	.Arg	(96, 160, 161)
Serum albumin (bovine)	Asp.Thr.	.(Leu,Ala,Thr).Ala	(105, 116, 118, 162)
Serum albumin (human)	Asp.Ala.	Gly.Val.Ala.Leu	(118, 162)
Silk fibroin		.Gly,.Ala(qual.) (2 chains)	(163)
Thyroglobulin	(qualitative: Thyroxyl., Diiodotyrosyl,Tyr., etc.)		(164)

* Uncertain residues are in parentheses. A period before any amino acid symbol indicates substitution of its amino group, and a period after the symbol substitution of the carboxyl group. Thus .Gly and Gly. mean —$NHCH_2COOH$ and NH_2CH_2CO—, respectively. Commas are placed between different chain ends of poly-chain proteins, and in parentheses between amino acid residues of uncertain sequence.

† Only traces (of the order of 1 residue per 300,000, or less).

‡ Upon treatment with 5 per cent TCA for 30 min. at 90°C., Pro.Ileu.Glu. appears as N-terminal sequence (145).

§ N-terminal residues of other globulins of other species, and of pathological globulins discussed in text.

N-terminal groups of serum globulins.—Determinations of N-terminal groups and sequence analyses have been applied with some success to normal and pathological globulins of man and other species during the past years. Urinary Bence Jones proteins were also analyzed and compared with the abnormal blood component (165, 166). Unfortunately, some authors publish only qualitative observations (167, 168). While skepticism is justified concerning the absolute significance of quantitative data obtained with the FDNB method, or any one method in this field, such data are of such distinct comparative value as to easily justify the small additional work involved.

The general conclusion of several laboratories is in accord with Porter's earlier findings (155b). Rabbit serum represents an exception in that its γ-globulin and several antibodies are comparatively homodisperse, all containing the same N-terminal pentapeptide and the same number of lysine groups (156, 157). In contrast, the human and equine γ-globulin fraction and several specific antibodies produced in these species represent mixtures of many molecular species characterized by different end groups (165, 166). Only in pathological cases, such as myeloma, does a single molecular species predominate, though differing from one individual to another. These pathological globulins, particularly the cryoglobulins which occur in many such cases, may be characterized by one or two N-terminal aspartic acid, from one to three glutamic acid, and one leucine residue, or a combination of aspartic and glutamic acid. According to Smith & McFadden, such proteins are not really abnormal components but indicate only abnormal rates of production of individual discrete components of the normal γ-globulin spectrum (75, 166). However, Putnam (165) holds the opposite vew. In most of these studies the different myeloma-globulins have also been characterized physicochemically, but in general, fewer differences become apparent in molecular weight or charge than in end-group analysis.

End-group of collagens, procollagen, and gelatin.—Collagen, procollagen, and gelatin have also been analyzed by several groups of workers (107, 108, 109, 152, 153). The more soluble fraction, procollagen, has apparently a very small number of N-terminal groups, and typical collagen has none unless it is degraded prior to analysis. On the other hand, Grassmann and co-workers (107, 108, 109) found an appreciable number of amino alcohols formed upon reduction; the yield amounted to about 5 per cent of all of the residues, or one thousand times the N-terminal residues of procollagen. Since the same amount was found with and without prior esterification, the authors concluded that these amino alcohols were derived from originally ester linked C-terminal amino acids, presumably involving the carbohydrate moiety of collagen. Their more recent finding that one-fifth of the C-terminal alanine of insulin is directly reducible (108) renders this hypothesis less necessary.

A number of peptides have been isolated by Kroner *et al.* from partially

hydrolyzed steer-hide collagen (169). The sequence Pro.Hypro was found to be of frequent occurrence, while the Pro.Gly.X sequence was infrequent.

According to Gustavson, the hydrothermal stability of different collagens is correlated with their hydroxyproline content (170, 171). He suggests that these groups are strongly hydrogen bonded. If such bonding involved the C-terminal carboxyl groups, and there is evidence against this, the latter might thereby be kept in the undissociated form and thus susceptible to direct $LiBH_4$ reduction without need for esterification, as observed by Grassmann. This interpretation would also be in accord with the finding of Veis & Cohen (172) that many carboxyl groups of collagen do not bind the basic dye, safranine, until the protein is heated to 60°C. for several hours; in contrast, the basic groups show the theoretical dye-binding properties. There remains the discrepancy, however, between numerous C-terminal and no N-terminal residues.

Elastin resembles collagen in being very poor in N-terminal residues, unless it is first degraded by elastase or acid (70).

Terminal sequence of tobacco mosaic virus.—Another protein which was intensively studied by two groups of workers is that of tobacco mosaic virus. The fact that the virus protein is built up of about 2800 probably identical protein subunits was established some years ago. The C-terminal position is occupied by threonine in all bona fide strains of tobacco mosaic virus that have been investigated (173). The finding of Harris & Knight that the dethreoninated virus, while retaining infectivity, shows slight but definite differences from the original virus in its immunological and electrophoretic behavior (H. K. Schachman) is noteworthy (174). Hydrazinolysis has established the same C-terminal tripeptide sequence (.Pro.Ala.Thr) for several strains (105). It remains to be explained why no trace of peptides was obtained in Berkeley from the virus, unless it be that the threonine, with apparently the most labile bond, was first enzymatically removed; in a subsequent study in Tübingen the theoretically possible di- and tri-peptides were found in direct hydrazinolysates of the virus protein (145). In a subsequent study by Niu & Fraenkel-Conrat the C-terminal hexapeptides were isolated from chymotryptic digests of several strains cf the virus, and the first sequential structural differences between strains was thereby revealed (143).

There now appears to be agreement that the virus protein contains no free N-terminal group. Only after treatment with hot trichloroacetic acid can proline be detected as the predominant N-terminal amino acid of the reaction product (175). Claims that a native protein fraction also contained N-terminal proline have been withdrawn (145). Similarly withdrawn was most of the severe criticism to which the Edman method had been previously subjected (175). A sequence of two amino acids next to the labile proline has been determined (.Pro.Ileu.Glu.) (145).

Unfortunately the evidence for cyclic peptides is of necessity largely

negative; the same is the case for various semi-cyclic peptides (a ring formed by amide linkage between one terminal and one side chain carboxyl or amino group). However, the more methods that are used in establishing the absence of an N- or C-terminal group, the more convincing becomes the case for a ring, or an ω-linked peptide. Thus, indirect evidence now strongly favors such a structure for the TMV-protein. Another protein in which a C-terminal amino acid sequence has recently been detected, but for which no N-terminal residue was ever found, is ovalbumin (105, 117). The collagen problem has been discussed above. Comparable evidence favors similar structures for chymotrypsin and trypsin, as well as for the corresponding zymogens, although in these cases it is the amino group which is free while no C-terminal groups have been detected (84, 105, 127 to 130, 149, 150, 151).

Structure of serum albumin.—Serum albumin from several species was found to contain one N-terminal amino acid (aspartic acid) and one C-terminal amino acid (105, 116, 118, 162); the latter, however, differed for different species. Complete reduction of all disulfide bonds and stabilization of the product by alkylation yielded a derivative of unchanged molecular weight (176). All this is in accord with the concept that this is a single-chain protein. In contrast, performic acid oxidation produced a product with a weight average molecular weight of 30,000 to 32,000 (osmotic pressure and light scattering) instead of the 66,000 to 77,000 for the original protein [Reichmannn & Colvin (177)]. This finding suggests the presence of a second cyclic chain attached by disulfide bonds to the open chain, and one would have to postulate that, in the work of McDuffie & Hunter (176), one disulfide bond resisted reduction. On the other hand the possibility of oxidative rupture of a single chain at a tryptophan or similar susceptible site does not appear to be excluded by the data of Reichmann & Colvin, even though those authors searched and failed to find a new N-terminal residue.

Methods for complete structural analysis.—Structural analysis of typical protein peptide chains of over 100 amino acids requires methods for the specific and quantitative splitting of certain bonds. Trypsin has proven a most useful tool for this purpose, since its requirement for a basic side chain on the amino acid contributing the —CO— group appears to be absolute. Only in one instance has it been suggested (178), on incompletely published evidence, that the enzyme "accepted" an ϵ-DNP-lysyl group instead of a basic side chain. Other evidence indicates that the ϵ-DNP-lysyl bond is resistant to trypsin (179). Chymotrypsin has shown great preference for phenylalanyl, tyrosyl, and leucyl bonds, while pepsin often splits, though not exclusively, the bonds on either side of acidic amino acids and phenylalanine. Subtilisin and papain show little specificity.

Earlier attempts to establish the specificity of the enzymes when acting on proteins have been handicapped by the lack of reliable methods for C-terminal analysis. Thus, attention was focused on the N-terminal groups

formed, and little selectivity became evident (179, 180). However, it seems that it is in general the C-terminal groups which are characteristically different as points of attack for each enzyme, and that more extensive use of C-terminal analysis will further establish the reliability of the enzymatic tools.

The hope that selective chemical methods would be found has not yet been fulfilled. Partial acid hydrolysis is occasionally used, but it will probably yield important information only in the case of smaller enzyme-resistant peptides. The specific release of aspartic acid in dilute acid has been reinvestigated by Blackburn & Lee (181), but this reaction has not yet been utilized in structural work.

For the separation and isolation of peptides from a protein digest many methods are now available, and best results are probably obtained with a combination of several of these. Countercurrent distribution has successfully been employed by Craig and co-workers, and by the Cyanamid group (28, 182, 183, 184) but appears to be a very laborious procedure for the separation of many peptides. Chromatographic separation on Dowex-50 X-2 has given clearcut separation of most of the peptides formed from ribonuclease [Hirs, Moore & Stein (185)] and has given characteristic patterns reminiscent of infrared spectra for digests from histones (186). Such "spectra" appear of great potential usefulness for the structural comparison of related proteins. Thus, the ion exchange method seems to show great promise. Other authors have relied largely on zone electrophoresis. Combination of this with paper chromatography is a powerful tool (187, 188). The separation of peptides, by any of these methods, in the form of their DNP-derivatives also is of great value (143, 182, 188).

Structural work on silk fibroin.—Silk fibroin (from *Bombyx mori*) continues to command the attention of chemists interested in protein structure, probably because its comparatively simple composition relates its properties to those of synthetic polymers. The simplest structure which has some support from analytical data and the isolation of peptides (189) is an alternation of glycine (G) with serine or alanine residues (X). This sequence GXGXGX is in accord with the x-ray diffraction patterns [Marsh, Corey & Pauling (190)]. A similar structure, allowing X to stand also for tyrosine and valine at regular intervals, seems to have been proposed by Ioffe (191).[4]

However, the results of Waldschmidt-Leitz & Zeiss (192) are not in accord with this structure. These authors isolated the chymotrypsin-resistant moiety of fibroin which had been degummed with papain, dispersed in lithium thiocyanate, and dialyzed. The product retained the typical x-ray crystallinity of silk fibroin, but was composed of only four amino acids, and was termed protofibroin by the authors. From the known specificity of chymotrypsin, tyrosine was expected to be the C-terminal amino acid, and this

[4] This paper was read only in abstract form.

was found to be the case by the thiohydantoin method and with carboxypeptidase [see also (163)]. Glycine and very little alanine were found to be N-terminal. From the peptides formed upon partial hydrolysis and the amino acid analytical data, the following structure was deduced: (Gly.Ala.Ser.Gly.Ala.Gly.)$_7$.Tyr. The isolation of peptides with adjacent alanine and serine residues, not previously found in degraded fibroin, is the key to this more complex structure.

The finding that Tussah silk fibroin, besides other sharp differences in the minor components, shows an inverted ratio of glycine and alanine residues suggests that the physical properties of silk are largely independent of the frequency of occurrence of the distinguishing methyl group (78). On the other hand, Braunitzer & Wolff (193) have recently investigated spider fibroin (*Nephila madagascariensis*) and found it remarkably similar to *Bombyx mori* fibroin, both in amino acid composition and in N-terminal residues; both types of fibroin contained a variety of end groups, amounting to only about 1 per 200,000 to 300,000 grams of protein.

The structure of ACTH.—With the help of selective enzymatic reactions, particularly trypsin and pepsin, and occasional use of partial acid hydrolysis, ACTH has been split into a sufficient number of overlapping peptide fragments to permit their sequential allocation to definite positions in the peptide chain. The isolation of the peptides was achieved by the methods discussed above. The amino acid sequences were largely established by the phenylisothiocyanate method, although carboxypeptidase and FDNB also played important roles.

These methods have enabled three laboratories to arrive, within about one year, at very similar, if not identical, sequences for the 39 amino acids comprising ACTH from both sheep and hog pituitaries. This was a particularly remarkable feat for the trail blazers who established sequences which had not previously been established [Bell and co-workers (28); White & Landmann (29, 30, 126); Harris & Li (124)].

The only differences of opinion, concerning hog ACTH, between the Cyanamid and the Armour group is the position of one aspartic acid (position 25 versus 28) and an amide group (194, 195). Sheep α-corticotropin seems to differ by one amino acid and there is uncertainty about the same area of the molecule which remains unsettled in hog ACTH (188).

Raacke & Li (196) have used starch electrophoresis to determine the isoelectric point of sheep corticotropin. After a detailed analysis of the theoretical aspects, and due compensation for electroosmotic flow, they arrived at the expected values for several reference proteins. However, the apparent isoelectric point of the hormone was pH 6.6 while from the amino acid composition a value of 9.3 was calculated. When similarly great discrepancies were found with certain enzymes by Velick and others, specific binding of phosphate by these proteins was shown to be responsible. No effects of such magnitude have been observed with monovalent buffers,

nor have Raacke & Li demonstrated any unusual binding affinities of α-corticotropin.

Ribonuclease.—The work on the threefold longer chain of oxidized ribonuclease has not progressed as far, but it is already a monumental achievement. Trypsin digestion has yielded 13 of the expected 15 peptides in near-stoichiometric yields (185). One peptide was found to contain two lysine residues, one of them apparently having resisted tryptic attack. Chymotryptic digestion has supplied a sufficient number of peptides to give "overlaps," thus establishing the sequential order of all but two of the trypsin fragments. These two are apparently near the carboxyl end of the chain (197).

Cytochrome.—Cytochrome-*c* represents another protein which is under active study in several laboratories. Tuppy & Bodo (198, 199, 200) again made use of the specificity of trypsin in producing a nonapeptide which carried the porphyrin and was thus easily separable from other colorless peptides in the digest. This peptide contained both cysteine residues, the anchors of the porphyrin, separated by two amino acids; a histidine residue was also present which is believed to be complex-bound to the iron.

A colored fragment produced by the action of pepsin on cytochrome-*c* had previously been described. Tuppy & Paléus have now found that a pure peptide can be isolated from such fractions; this extends the known area by three more amino acids (201). The specificities of trypsin and pepsin are again borne out in the observed points of attack:

```
                  |              |
                  S              S
                  |              |
  Val.GluNH2.Lys.Cy.Ala.GluNH2.Cy.His.Thr.Val.Glu.Lys.
  Trypsin        ↑                                    ↑
↑ Pepsin                                         ↑
```

An interesting observation is that chicken cytochrome-*c* differs from the salmon, beef, sheep, and cow enzyme by containing a serine in place of the alanine residue between the two cysteine residues (202). The enzymatic split products of cytochrome-*c* have interesting catalytic properties, a study of which may contribute to an understanding of the more complex activity of the intact enzyme (203). The spectrum of the nonapeptide resembled that of cytochrome-*c* if histidine was added to the solution (200). A model of the hendeca-peptide obtained with pepsin and the attached heme was constructed by Ehrenberg & Theorell (204). An α-helical structure was in accord with the data, and would permit both the imidazole and the lysine amino group to be complexed to the iron. The tendency of the peptide to aggregate above pH 7.0 to a molecular weight of about 11,000 was studied under various conditions and described in terms of its structure.

Margoliash has attacked the problem of the structure of cytochrome-*c* by means of the FDNB reaction (131). He believes that he has found evi-

dence that each of the four histidine residues of cytochrome-*c* shows characteristic behavior. One is N-terminal, but masked in regard to the reactivity of its imidazole group; this would represent the second iron-complexed group of the protein. Another N-terminal histidine is free to give di-DNP histidine. Of the two internal residues, one has a masked imidazole group, presumably that occurring in the enzymatically obtained peptides, and one is free to react. These results were not reproducible in preliminary experiments of the reviewer.

Paléus has corrected earlier analyses of the sulfur-containing amino acids of cytochrome-*c*. The protein contains only two methionine residues, besides the two cysteines which carry the prosthetic group (205).

Lysozyme.—Lysozyme is another protein which lends itself to structural analysis by present methods. A great number of peptides have been isolated and characterized from partial acid hydrolysates of this protein (206, 207), but allocation of their position will have to await the application of methods yielding bigger peptides. This only illustrates the obvious fact that the methods which were most useful for the elucidation of insulin will be inadequate for longer peptide chains.

Some of the other proteins in which isolated peptide sequences have been established are ovalbumin and pepsin [phosphorylated and cysteic acid peptides (208, 209)], trypsinogen and chymotrypsinogen [arginine and cysteic acid peptides (210, 211), peptide formed in the course of enzyme activation, and adjacent sequences (84, 130)], and papain [cysteic acid peptides (212)].

The structure of insulin.—Sanger and co-workers put the finishing touches to elucidation of the chemical structure of insulin when the positions of the amide groups (213) were determined and the more difficult allocation of the cross-linking disulfide bonds was achieved (187).

The main difficulty encountered in this work was attributable to the great tendencies of disulfides to undergo interchange. Thus, considerably more peptides occurred in degraded insulin than could have been present in the original protein. Through the work of Ryle & Sanger (214) two different modes of disulfide exchange were established. In neutral or alkaline solution, traces of thiols catalyzed, and thiol-blocking agents prevented, exchange. On the other hand, in strong acid, thiols had the opposite effect, and their presence (about 5×10^{-3} *M*), combined with the use of 10 *N* H_2SO_4 for 2 hr. at 100°C., or for 10 days at 37°C., yielded hydrolysates containing no appreciable amounts of rearranged peptides (187).

The Cambridge workers also determined the structural differences of insulin from three different species (4). High voltage paper electrophoresis proved a powerful tool in the separation of cysteic acid peptides and other peptides encountered in this work. It appears that all differences are located in the A chain and within the ring of four amino acids formed by the two half-cystine residues. Thus the amino acid sequence of the eighth to tenth

residue is .Ala.Ser.Val. for cattle, .Thr.Ser.Ileu. for pig, and .Ala.Gly.Val. for sheep insulin. It must be noted that these differences are similar in nature and location (between two half-cystines closing a ring) to the difference detected in cytochrome-*c* from different species (202).

The question of the molecular weight of the insulin monomer (about 6000) appears now to be settled beyond doubt [Kupke & Linderstrøm-Lang (215)], but there is also good evidence for the specificity, firmness, and structural significance of a dimeric form and, at least in the case of zinc-insulin, for higher states of aggregation (216).

Sluytermans (217) has applied the principle of Craig's partial substitution technique to the molecular weight problem. He prepared partly acetylated insulin by the use of small amounts of acetic anhydride at 0°C. and at neutrality. By means of paper electrophoresis in 33 per cent acetic acid a maximum of three acetyl derivatives could be detected in such reaction mixtures. If one assumes the separation method to be adequate to the task of resolving five or six components within the same range of charges, then the finding of three components is an indication that under the conditions of electrophoretic separation each molecule contained only three original amino groups, and that the molecular weight was, therefore, that of only one A chain and one B chain (about 6000). Evidence was also adduced that no disaggregation occurred under the conditions of acetylation.

A similar study was performed by Andersen with phenylisocyanate, generated *in situ*, as the reagent (218). Unreacted insulin and its mono- and di-substituted derivatives were isolated by partition chromatography. The N-terminal residues were separated and determined as their phenylhydantoins. The monoderivative proved to be largely glycine substituted, but the rate of introduction of the second phenylcarbamyl residue was much faster than that of the reaction of phenylalanine in the native protein. This suggests a vicinal location of the two N-terminal groups, in accord with the model of parallel chains proposed by Lindley & Rollet (219).

Insulin may be the first protein in which the steric arrangement of the peptide chains in the native molecule will be established. A model has been built by Lindley on the basis of a partly left- and partly right-handed α-helix which accommodates the disulfide bridges and appears to fit all requirements and to allow for a maximum number of hydrogen-bonded peptide bonds (219). This is in accord with data on optical rotation, exchangeability of hydrogen with deuterium, and resistance to digestion by papain, in all of which respects the behavior of native insulin differs markedly from that of the oxidatively separated A and B chains (102, 220, 221, 222).

Lindley also has studied the reductive cleavage of insulin with thioglycolate at pH 5 (223). In 8 *M* urea he was able, in contrast to earlier investigators, to achieve complete reduction and to separate the two reduced chains by paper electrophoresis. This technique, if it is generally applicable, has great advantages over the oxidative method of removal of disulfide

crosslinks in proteins generally, since it does not damage tryptophan, tyrosine, and methionine residues. Lindley states that in native insulin only the intrachain disulfide bond on the A chain is readily reducible, but the evidence for this conclusion appears insufficient. If the most readily reducible bond is really that closing the above mentioned pentapeptide ring, then its integrity would appear necessary for the hormonal activity of insulin, as a disulfide ring of this size is for the activity of the posterior pituitary peptides. One might believe, a priori, that this ring should be reversibly reducible in insulin as it is in the peptide hormones, resulting in reversible inactivation. That this is not the case is explained by Lindley on the basis of strain, since the configuration of the A chain in this area is dependent on this bond being intact. A contributing factor in the nonreversibility of the reduction may well be the ease with which, according to recent investigations (224), sulfhydryl bonds can exchange hydrogen with available disulfide bonds (see disulfide exchange discussed above) both intra- and intermolecularly, thus rapidly causing havoc with the structural integrity and homogeneity of such a preparation.

Protein structure and biological activity.—The importance of disulfide bonds as compared to hydrogen bonds in maintaining protein structure is at present under active discussion. Anfinsen *et al.* compared the rate of peptide deuterium exchange, and the rate of change of viscosity and optical rotation which occur in ribonuclease in 8 *M* urea, with those produced by oxidative cleavage of all disulfide bonds (225). From similarities in these changes, the authors conclude that the peptide chain is held in an orderly, presumably helical, arrangement largely by hydrogen bonds and that the disulfide bonds may supply rather short-range bridges in this protein. A corollary of this conclusion is that the "active site" of ribonuclease (and other enzymes) may be small and largely independent of the shape of the molecule as a whole. However, insulin resembles ribonuclease in its behavior in urea [Hvidt (226)], and yet the important structural role of the disulfide bonds in insulin is well established. According to Hvidt . . . "the H-bonded structure in native ribonuclease seems to be greatly stabilized by the —S—S— bridges. . . . " It seems advisable to distinguish clearly those relatively small proteins which are generally very rich in cystine and which show only completely reversible effects of urea from those bigger proteins which are irreversibly denatured by urea. It appears that the behavior of such proteins as ribonuclease and lysozyme is most easily explained on the basis of a structure which is stabilized by well placed disulfide bonds to such an extent that the loosening of the structure produced by denaturants, such as urea, is spontaneously reversed upon their removal. This question will probably be solved only when the amino acid sequence of ribonuclease has been sufficiently elucidated to permit allocation of the disulfide crosslinks.

In a recent study of the reaction of TMV with iodine (see p. 316), a sulfenyliodide group was found to be the final product. Since simple sulfenyl-

iodides are quite unstable in aqueous solution as well as in the pure state, this finding illustrates a new property of proteins; their hydrogen-bonded lattice structure, which accounts for the masking of —SH and other groups, apparently can stabilize organic reaction intermediates which are normally quite unstable. It was proposed that this might be of great functional importance in enzymatic processes [Fraenkel-Conrat (227)]. Recent evidence is accumulating which strongly suggests that in the action of most esterases and proteases, such a stabilization by the native protein of a normally labile bond plays a decisive role. The extensive literature on the inhibition of these enzymes by reactive dialkyl phosphates was well reviewed by Schwert last year (228) and will be brought up to date by Fruton in this volume (p. 57). Here, therefore, only some of the recent evidence bearing on the protein-chemical mechanism of that inhibition will be discussed.

On the basis of comparative results obtained with *p*-nitrophenyl-phosphate, -acetate, and -carbonate, Hartley & Kilby (229) have proposed a beautifully illustrative scheme which has been further supported by recent kinetic data [Hammond & Gutfreund (230)].

$$\mathrm{E{-}H + A{-}B \underset{I}{\rightleftharpoons} \underset{\displaystyle A{-}B}{\underset{|}{E{-}H}} \underset{II}{\rightarrow} E{-}A + B{-}H}$$

Reaction I II

$$\mathrm{E{-}A \xrightarrow[III]{H_2O} E{-}H + A{-}OH}$$

Reaction III

At first all types of suitable esters (A—B) are rapidly bound to the reactive site (E—H) (Reaction I). They are then more slowly split, with the acyl moiety forming a primary bond with the enzymatic site (Reaction II). In the case of the diakyl phosphate esters, the substituted phosphate is so stably bound that it will not be released at a significant rate by the native enzyme, and complete inhibition ensues (rate of Reaction III$\cong$0). In contrast, the acetate, rapidly introduced in similarly stoichiometric manner by *p*-nitrophenyl acetate, is slowly released and thus acts both as inhibitor and substrate. For typical substrates, the rate of release of the acyl residue (Reaction III) must be quite rapid. It seems that all known facts about this reaction are in accord with such a scheme.

Recently, Balls & Aldrich have isolated and crystallized the intermediate mono-acetyl chymotrypsin (A = acetyl) formed from interaction with any of the nitrophenyl acetates and studied its lability in some detail (231). The nature of the reactive group in the enzyme is naturally of greatest interest. It is obviously not a serine hydroxyl group for serine O-acetate and phosphate are much too stable to account for the behavior of the system. Balls considers an —SH group, which might well give such "high energy" acyl derivatives, but concludes his article as follows: "All that can be said at present is that our efforts to demonstrate the existence of an essential sulf-

hydryl group in chymotrypsin have failed so consistently that we are beginning to doubt its existence."

Several independent pieces of evidence had previously suggested that an imidazole group was connected with the activity of this class of enzymes [summarized by Desnuelle (232)]. In the light of the evidence cited earlier concerning the stabilizing effect of protein structure on active intermediates, a "masked" histidine group could well represent the site of attachment, of varying stability, of the phosphate, acetyl, or peptidyl group. To test this hypothesis, experiments were initiated in the reviewer's laboratory to compare the reactivity towards FDNB of native chymotrypsin and of its diisopropylphosphoryl derivative. The results indicated that one of the two histidine residues in the inhibited enzyme showed decreased reactivity, while the tyrosine and lysine groups reacted at similar rates in the two proteins (233). Thus, direct experimental evidence is now at hand suggesting that an imidazole group is the site of attachment of the inhibitory phosphate, and thus most probably an important part of the enzymatic site. The same conclusion was recently reached by Gutfreund for chymotrypsin and trypsin on the basis of similarities between the imidazole groups and the catalytic site in regard to their ionization constants as affected by temperature (234). While this approach was certainly meaningful in this particular case, a note of warning is here necessary. Protein groups do not necessarily show the same pK values, as the same groups in simple peptides, and no conclusions should be based on titration curves as sole evidence (216, 235).

Unusual bonds in proteins.—The classical concept of protein structure allows only for α-peptide linkages and disulfide bonds. There is, however, definite evidence for the occurrence of other bonds in proteins or other natural peptides, and presumptive evidence for others is accumulating.

Evidence has been adduced that phosphate groups may form crosslinks both as diesters and as pyrophosphate diesters (236, 237). Indirect evidence for ω-peptide linkages (involving an α-amino or carboxyl group) has previously been reviewed. Direct evidence, though circumstantial, was presented from Bruckner's laboratory which suggests the existence of such bonds (238, 239). These authors subjected insulin, gliadin, and chymotrypsin to the Hofmann degradation and found in the hydrolysates of the former two proteins, some formyl propionic acid which would be expected to be formed only from isoglutamine in peptide linkage.

$$\mathrm{R\cdot CO\cdot NH\cdot CH(CONH_2)\cdot CH_2\cdot CH_2\cdot CO\cdot NH—} \xrightarrow{\mathrm{KOCl}}$$

$$\mathrm{R\cdot CO\cdot NH\cdot CH(NH_2)\cdot CH_2\cdot CH_2\cdot CO\cdot NH—} \xrightarrow{\mathrm{H^+}}$$

$$\mathrm{[NH_2\cdot CH(NH_2)\cdot CH_2\cdot CH_2\cdot COOH] \rightarrow O : CH\cdot CH_2\cdot CH_2\cdot COOH}$$

The same technique had previously given the expected results with γ- and α-polyglutamic acid, but the authors are cautious in the interpretation of their results in view of the complexity of the system.

The evidence for the occurrence of ester bonds in collagen has previously been discussed. Interchain amide bonds have often been considered, but never proven. From performic oxidation experiments with α-chymotrypsin Keil concludes that bonds other than disulfide, and probably peptide bonds, must hold the two chains together (240).[4]

An oddity which remains unexplained is that recent analyses for the sulfur-containing amino acids, performed by the most up-to-date method (241) and in the very best laboratories, fail to account for all the sulfur in two of the most intensively studied proteins of the moment (77, 79). This suggests the presence of unusual linkages or constituents in these proteins.

Unusual structures in natural peptides.—An immense amount of structural work on antibiotics is going on which is beyond the scope of this chapter. However, the proposed structures of a few peptide-antibiotics will briefly be reviewed, because they may open our eyes to possibilities which we protein chemists generally prefer not to face.

Bacitracin A (Fig. 1) is a peptide which appears to contain a number of the unusual bonds which have yet to be demonstrated in proteins. The structure suggested by much painstaking work, mainly of two laboratories, contains one peptide linkage with the ϵ-amino group of lysine, and another involving the γ-carboxyl of aspartic acid. There also is evidence for an—SH group, "masked" through closure of a thiazoline ring between a cysteine and an adjacent isoleucine residue, a ring which strongly affects the reactivity of the amino group, and enables it to form a bond with a phenylalanine residue [Weisinger, Hausmann & Craig (183, 184, 242); Lockhart & Abraham (243)]. The nature of this bond is almost beyond conjecture.

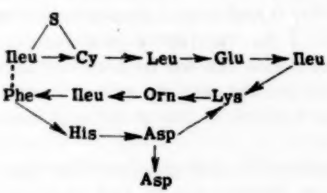


FIG. 1. Proposed structure of bacitracin A. (Arrows denote the direction of the —CO—NH— bond in cyclic peptides.)

The structure of tyrocidin A, elucidated by the jigsaw-puzzle technique by Paladini & Craig (182), appears simple compared to that of the bacitracins but has presented a real challenge to the ingenuity of the investigators, particularly in regard to locating the two D-phenylalanine residues characteristic of this antibiotic. The following structure is proposed for tyrocidin A

cyclo(.L-Orn → L-Leu.D-Phe.L-Pro.L-Phe.D-Phe.L-Asp.L-Glu.L-Tyr.L-Val.).

Another peptide of unusual interest is phalloidin, a mushroom toxin. The

final elucidation of its structure had to await the identification of a new amino acid of unusual properties, termed δ-oxyleucenin (abbreviation: Oleu) by Wieland & Schön (244)

$$(CH_2OH \cdot C(OH)(CH_3) \cdot CH_2 \cdot CH(NH_2) \cdot COOH)$$

The second unusual feature of this cyclic peptide is an —S—C-bond between the cysteine and the α-position of the indole group of tryptophan which makes this a bicyclic peptide with a new type of sulfur crosslink, which certainly would escape detection if it occurred in proteins. Upon acid hydrolysis of phalloidin, hydroxytryptophan and cysteine are formed and the δ-oxyleucenin is transformed to, and isolated as, the lactone of γ,δ-dioxyleucin The complete structure is believed to be that shown in Figure 2 (244). The data obtained in Sorm's (245) laboratory appear to be in accord with these conclusions.

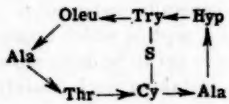


FIG. 2. Proposed structure of phalloidin.

PROTEIN DERIVATIVES

Elucidation of the mode of action of simple biologically active organic compounds usually relies on both structural analysis and the study of derivatives. With proteins, emphasis has in recent years been largely on structural analysis. However, as amino acid sequences become established without revealing the secrets of the "active" region, interest is returning to the no-less-inconclusive evidence derived from substitution or modification by means of selective reagents. As yet only a small fraction of the time allotted to structural analysis is given to the study and improvement of such reagents.

Reaction of imidazole and indole groups.—The approach through derivatives has yielded most striking results and conclusions in the previously discussed interaction of esterases with dialkyl-fluorophosphates and related agents.

In a study of the effect of peroxidase and H_2O_2 on chymotrypsin Wood & Balls found that a derivative of about half the original activity resulted concomitantly with uptake of one mole of oxygen and destruction of one residue of tryptophan but not of histidine or tyrosine (246). Reactivity to, and inhibition by, diisopropyl fluorophosphate was unimpaired. These results, in conjunction with those earlier obtained by Weil with photooxidation, were in accord with the idea that a histidine residue was involved in the dialkyl phosphate-binding by the enzymatic site, but they also suggested the presence of an indole group near that site. It now seems possible

that a half-active periodate oxidation product that had been crystallized by Jansen and co-workers in 1951 (247) also was modified at the same indole group. The other finding of that earlier study, an apparent loss of about 25 per cent of the enzymatic activity upon acetylation of many amino groups (by means of acetic anhydride), also gains new significance in the light of recent preparation (by means of nitrophenylacetate) of a mono-acetyl derivative which regains activity as it loses its acetyl group under the conditions of assay (231). The evidence for the existence of a particular "semi-masked" imidazole group representing the site of attachment of the diisopropyl phosphate, and presumably also the acetyl or substrate peptidyl group, has previously been reviewed.

Recent work by Massey & Hartley (248) indicates that a fluorescent dye, as the sulfonyl chloride, may also be introduced into the active site; these authors have likewise attempted to use FDNB in conjunction with various inhibitors of chymotrypsin, and their results favor the idea that an imidazole group is involved (249). Recent work by Weil indicates that ribonuclease also contains an essential imidazole group (250). With this protein, which is free of tryptophan, histidine represents the only amino acid susceptible to appreciable photooxidation under the experimental conditions, and a comparison of the rates of inactivation and of disappearance of the histidine definitely shows the crucial importance of at least one, and probably only one, of the four histidine residues of this protein.

Reaction of amino and carboxyl groups.—Selective acetylation of the amino groups of proteins by means of acyl anhydrides in neutral aqueous solution at 0°C. continues to be used in structural and enzymatic studies. The preparation of partly acetylated insulin has previously been described (217). In a study of the mode of inactivation of TMV by formaldehyde, acetylation has supplied part of the evidence that the reaction with the nucleic acid, rather than with the protein moiety, was responsible (251). The enzymatic properties of acetyl-trypsin have been reinvestigated in some detail, as well as its greatly decreased affinity for trypsin inhibitors (252). On this basis higher acyl-trypsins have been advocated for medicinal purposes (253).

The effects of acetylation of various antigens and the corresponding antibodies on immunological reactions have been studied by Marrack & Orlans (254). It became evident that the amino groups played no key role in most of these protein interactions. The observed quantitative differences were attributed to changes in charge, and thus in structure.

Acetylation had no effect on the binding of testosterone by serum albumin, in contrast to the binding of anionic compounds [Levedahl & Bernstein (255)]. Esterification of serum albumin was critically reinvestigated by Saroff and co-workers (256). These authors confirmed the specificity of methyl alcoholic HCl, in contrast to methyl sulfate. They also found no evidence for denaturation of the protein.

As part of a detailed physicochemical investigation by Theorell, Nygaard, and others, of the binding of the prosthetic group, flavin mononucleotide, with the old yellow enzyme, the effect of protein modifications has also been surveyed. It seems that blocking of amino and phenolic groups prevents this interaction; the phosphate groups of the nucleotide are believed to require amino groups for ionic attachment, and the isoalloxazine ring to be anchored through hydrogen bonds to tyrosine and possibly also other groups (257, 258)

Reactions of —SH and —S—S groups.—The reinvestigation of the reaction of TMV with iodine has previously been mentioned because of its protein-structural implications. Possibly because only a single —SH group occurs in each protein subunit of the virus this group was found not to become oxidized to a disulfide but to undergo two-step oxidation to a sulfenyliodide. Although no other proteins were found to give stable sulfenyl iodide groups, these or the corresponding sulfenic acids were postulated as likely intermediates in the oxidation of sulfhydryl groups generally (227).

An unrelated finding by Klotz and co-workers also deals with the single —SH group of a particular protein, and yet has important general implications (259). These workers found spectrophotometric evidence for a specific binding of copper to the —SH group of bovine serum albumin, but of no other -SH protein including human serum albumin. They found further that this copper complex decomposed with time, most probably by involving a vicinal disulfide bond. The specific triggering —SH group which led Klotz *et al.* to this discovery is most probably not necessary for the secondary slow effect, since they found simple disulfides to become hydrolysed at neutrality in the presence of a trace of copper. This reaction may well serve to explain the sensitivity to copper of the otherwise stable disulfide proteins, lysozyme and ribonuclease (260, 261). Chain reactions of the disulfide exchange type may again be involved. The correlation noted by Setlow between the ultraviolet sensitivity of proteins and their cystine content may be a related phenomenon (262).

Recent experiments achieving, for the first time, a complete reduction of the disulfide bonds of insulin have been previously discussed (223). The reactivity of the disulfide bonds of wool have also been reinvestigated (263). Reduction and alkylation of the "reactive" disulfide bonds, renders additional —S—S bonds susceptible to reduction, an indication that —SH groups may stabilize protein structures, presumably through hydrogen bonding.[5] Reduction-alkylation of serum albumin was previously mentioned (176).

The reactivity of the —SH groups in proteins has been studied by various

[5] A particular hydrogen-bonding role of —SH groups in proteins was postulated by the reviewer some years ago on the basis of the known tendency of completely reduced proteins to form aggregates. The masked state of many —SH groups in proteins was more readily explainable on that basis. More recent studies by Benesch on the reactivity of the —SH group of cysteine-peptides are in accord with this view.

methods. The use of "tris buffer" during amperometric titration has rendered this a method well suited for —SH analysis in proteins. Partly confirmatory and partly new and interesting results were obtained by the Benesch's with egg and serum albumin, various enzymes, and hemoglobins (264). Concordant, somewhat more extensive, data with hemoglobin were obtained by Ingram by the combined use of amperometric silver titration and other —SH reagents of higher affinity (e.g., mercurials) (265). A convenient extension of the usefulness of *p*-chloromercuribenzoate has been developed by Boyer. It consists in observing the spectrophotometric effect of mercaptide formation (266).

An unusual difference in the affinity of —SH groups for related specific reagents was observed with yeast glyceraldehyde phosphate dehydrogenase. This enzyme lost its enzymatic activity and capacity to bind diphosphopyridine nucleotide upon addition of two equivalents of *p*-chloromercuribenzoate, but required for such inactivation four equivalents of methyl mercuric bromide, enough to react with all four available —SH groups. When only two methyl mercaptides had been formed, 74 per cent of the activity remained [Halsey (267)]. It might be noted that at least one of the —SH groups of this enzyme appears to belong to the class of "semi-masked" protein groups which can stabilize reaction intermediates (see earlier discussion). Thus this —SH group appears to bind alternately diphosphophyridine nucleotide and an acyl (glyceryl) residue in the course of enzymatic function [Krimsky & Racker (268)].

Two other unconventional —SH proteins (one begins to doubt the existence of the conventional norm) are phosphorylase-*a* and -*b*. According to Madson & Cori (269), phosphorylase contains all its cyst(e)ine in the form of reactive —SH groups, and all 17 or 18 are "essential" in the sense that inactivation by *p*-chloromercuribenzoate gradually approaches 100 per cent as the stoichiometric amount of the reagent is added and bound (as measured spectrophotometrically). At this point the s_{20w} is reduced from 13.2 to 5.6 (expressed as *S*) which suggests, a quartering of the enzyme molecule. Upon addition of cysteine, all effects are reversible. Phosphorylase-*b* acts similarly. These experiments suggest that four subunits of the enzyme are held together only by secondary forces involving the —SH groups.[5] The reversibility of this disaggregation appears particularly remarkable since the original bonds must, presumably, be exactly reformed to reproduce the original enzymatic and physicochemical properties.

The role of trace amounts of —SH in causing instability in disulfide proteins through chain-reacting disulfide exchange (see above) has been held responsible by Burley for the long-range elasticity of wool. Evidence for this was the decreased extensibility of wool when treated with —SH blocking reagents or when stretched in acid solution (270). Undamaged wool contained about 10μM. of —SH per gm., and that from copper-deficient sheep almost three times as much, when determined with Bennett's reagent (271).

Analyses performed in Zahn's laboratory by a new method, i.e., isolation of the S-DNP derivative of cysteine, yielded a similar value (272).

The reactions which lead from fibrinogen to a fibrin clot continue to represent a challenging problem. From papers by Mihalyi (273, 274), and Loewy & Edsall (275), and others which appeared during the past two years, the following oversimplified picture seems to emerge. Fibrinogen undergoes limited proteolysis under the influence of thrombin and yields "activated" fibrinogen and an acidic peptide. The latter contains a new type of protein constituent, a tyrosine-O-sulfate residue [Bettelheim (276)]. The activated fibrinogen aggregates to a gel which resembles the clot but can be distinguished from it by its solubility in such disaggregating solvents as urea. This potentially soluble fibrin, termed fibrin *s*, forms a three dimensional network through hydrogen bonds between imidazole, tyrosine, and probably other groups. However, in the presence of Ca ions or a nondialysable factor first described by Lorand & Laki, fibrin *s* is transformed to fibrin *i*,—insoluble in urea and similar solvents. This last reaction is most probably of the same sulfhydryl-disulfide exchange type which has been repeatedly mentioned in this review. Thus a three dimensional network of primary bonds is formed through a chain reaction, involving probably very few —SH groups, but many reactive disulfide bonds (277) which are gradually transformed from intramolecular to intermolecular crosslinks. The inability of several authors to find —SH groups is thus not too incongruous (277, 278[4]). The factors and reaction conditions needed for the fibrin *s* $\rightarrow$ fibrin *i* transformation are as yet poorly understood, but it would appear that they must uncover the few —SH groups needed as the triggger for the chain reaction.

An end group analysis of the plasma clot by Middlebrook indicated the appearance of the same six chain ends which are formed from purified fibrinogen under the influence of thrombin (two N-terminal tyrosines, four glycines). However, one of the glycine chains does not take part in the crosslinked network and can be extracted from the clot with urea; it is then dialysable (93).

The dissociation constant of the —SH group of cysteine has been reinvestigated in several laboratories. The idea first expressed by Edsall that the secondary and tertiary inflections of the titration curve of the amino acid are composite dissociations of the —SH and the $—NH_3^+$ groups is supported by experimental results obtained by Grafius & Neilands (279).

Miscellaneous reactions.—Wool has also been subjected to phenylisocyanate and other reagents for the purpose of modifying its physical properties (280, 281). 1,5-Difluoro-dinitrobenzene, as well as the chloro-fluoro-derivative, have been used as crosslinking agents (282). More interchain crosslinks, particularly between tyrosine and lysine residues, are formed than might be expected. From this Zahn concludes that there are areas in which these groups are concentrated (283). This is in accord with present concepts of the structure of fibrous proteins. The novel use of phenylisocya-

nate for end group analysis has previously been discussed (218). Periodate has been used as a reagent for the detection of N-terminal serine (284). Vigorous treatment with this reagent was found to destroy the tryptophan and the —SH groups of ovalbumin, prior to attacking other residues (285).

The guanidylation of proteins by means of S-methyl thiourea in ammonia solution has been reinvestigated by Roche *et al.* (286). Transformation of the lysine to homoarginine residues was quantitative in most proteins studied, but no evidence for reaction of N-terminal residues was detected. This interesting specificity suggests that it is the dissociated form of the amino groups which enters into the reaction. The incompleteness of the reaction in some proteins, notably gelatin, could be explained on the same basis. When the guanidylation was carried out at pH 10 to 12 with O-methyl isourea, the lysine and hydroxylysine of gelatine reacted quantitatively. The resulting basic amino acids were well separated by the Moore & Stein procedure [Eastone & Kenchington (287)].

Another reaction which has often been used but not studied critically is the coupling with diazo compounds. The present investigation from Klotz' laboratory shows that this reaction is much less specific than was anticipated (288). Many more reagent molecules were bound than diazo tests and colorimetric analyses showed to be coupled as azo compounds.

A very extraordinary reaction, first observed in the course of elucidation of the structure of oxytocin and vasopressin, has been studied in more detail by Ressler & du Vigneaud (289). It appeared that bromine water brominated the tyrosine residue of these peptides and concomitantly caused a selective hydrolysis of the tyrosyl-peptide linkage. When bromination was performed in glacial acetic acid, no splitting of the peptide resulted; when bromination of the phenolic group was prevented by its prior dinitrophenylation, then bromine water caused no peptide split.

Performic acid is now commonly used for the oxidation of cystine to cysteic acid (241). During this reaction tyrosine has at times been unaccountably affected. The final reaction products, after HCl hydrolysis, have now been recognized by Thompson (290) as 3-chloro- and 3,5-dichloro-tyrosine. Conditions were also described which would tend to avoid this side reaction.

In conclusion of this section, the reviewer notes with regret the absence of published evidence of any search for better methods needed so badly for selective modification of protein groups other than amino and sulfhydryl.

Synthesis

Methods.—The methods used for the synthesis of peptides in 1955 have been generally the same as those used in preceding years. An innovation is the suggested use of nitrophenyl esters as a means of activation of the carboxyl group. According to Bodánszky, ethyl glycinate treated with any of the nitrophenyl esters of phthalyl glycine gives higher yields of phthalyl

diglycine ethyl ester, than when treated with the thiophenyl ester (291). The nitrothiophenyl esters, suggested by Farrington, may be even better. Thus the relative rates of reaction with an amine were 1:16:140 for thiophenyl, *p*-nitrophenyl, and *p*-nitrothiophenyl esters, respectively (292).

The use of carbodiimide as a condensing agent for acyl amino acids and amino acid esters has been advocated by Sheehan & Hess (293, 294) and used in the preparation of nonpolar peptides as well as of serine and threonine peptides. However, according to Khorana, who has much prior experience with carbodiimides, side reactions are likely to present a serious problem (295).

Synthesis of natural peptides.—The fact that peptide-synthetic methodology has made no major advances may be regarded as an indication that adequate techniques are now available for the job at hand. In any case, recent years have witnessed the first complete syntheses of nonapeptide hormones and a beginning in the synthesis of a 39-residue peptide hormone, both within one to two years after elucidation of their amino acid sequence.

The brilliant series of syntheses by du Vigneaud and co-workers which first produced pure oxytocin (296) has now led to the preparation of seemingly pure arginine vasopressin (297). The corresponding open nonapeptide derivative was obtained by the pyrophosphite method from the protected pentapeptide Cy(S-benzyl).Tyr.Phe.GluNH_2.AspNH_2 and the tetrapeptide Cy(S-benzyl).Pro.Arg.GlyNH_2. The former was derived from the protected Cy(S-benzyl).Tyr and Phe.GluNH_2ASpNH_2 by the mixed anhydride method. The tetrapeptide was prepared from the two dipeptides Cy(S-benzyl.Pro and Arg.GlyNH_2 by pyrophosphate condensation. The *p*-nitro derivative of carbobenzoxychloride, proposed by Carpenter & Gish, was employed as a protecting group in the preparation of the tetrapeptide. The open nonapeptide was transformed to the hormone by reductive release of the —SH groups and oxidation by air to the cyclic disulfide. The product was purified by countercurrent distribution. It corresponded to the natural hormone in all biological activities tested, and in its partition coefficient, electrophoretic mobility, and ion exchange behavior.

The previously described synthesis of oxytocin led to a product that was identical with the natural hormone in all these properties, and, probably for old times sake, showed no mixed melting point depression (296). The key in that synthesis was the heptapeptide amide (Ileu.GluNH_2.AspNH_2.Cy(S-benzyl).Pro.Leu.GlyNH_2) which was finally condensed with the protected Cy(S-benzyl).Tyr. moiety. The heptapeptide, as well as the two intact hormones, showed high strepogenin activity, which decreased when peptides shortened by one or two residues from either end were tested (47).

Synthetic work on ACTH has been described by Hofmann & Jöhl which confirms the N-terminal pentapeptide sequence (298). Since this is the part of the molecule which is beyond doubt identical in sheep and hog, which is probably essential for hormonal activity, and which is also quite problematical in its behavior towards FDNB, this synthetic confirmation is of great

importance. The authors used both the mixed anhydride method and azides for activation of the carboxyl groups. The pentapeptide which they reported on (Ser.Tyr.Ser.Met.Glu) was identical with that obtained by the Armour group in its chromatographic behavior in several solvents and in its susceptibility to carboxypeptidase and aminopeptidase.

Preparation of amino acid polymers.—The preparation of amino acid polymers from their α-N-carboxyanhydrides continues to represent a fertile field of investigation. Recent additions to this series are poly-L-tryptophan and poly-L-proline (299, 300). It appears of interest that the latter is water-souble, in contrast to all other nonpolar amino acid polymers, except those formed from sarcosine and D,L-alanine. It seems that the —CONH— group, through its hydrogen bonding, accounts for the insolubility of the polymers and that the racemic amino acid does not permit sufficient close packing. Interesting investigations of the mechanism of the termination reactions and a theoretical analysis of the polymerization have also been published from Katchalski's laboratory (301, 302). It appears that the amine-initiated polymerization is terminated with a urea-linked carboxylic acid, thus leading to polymers with two terminal carboxyl groups

$$HOOC \cdot CHR \cdot NH \cdot CO[NH \cdot CHR \cdot CO]_n\text{-}NH \cdot CHR \cdot COOH.$$

Techniques for titration of the end groups were described in detail, as well as a method to determine the hydantoins formed from the terminal two amino acids at the urea end.

While hardly any amino acid polymers remain to be prepared, the field of copolymerization has barely been touched, and the possibilities there are just about unlimited. A particular stimulus to the study of copolymers is the remarkable finding of Katchalski *et al.* (303) that antibiotic activity related to that of gramicidin S is shown by a number of copolymers of the amino acids characteristic of that peptide, cyclo (L-Val.L-Orn.L-Leu.D-Phe-L-Pro.)$_2$. Quite surprisingly, however, only two factors appeared essential for this antibiotic activity: the ornithine with its free amino group and four other amino acids as "diluents." Thus, open chain polymers of various combinations of several D,L, or racemic amino acids were found active, as long as they fulfilled the above requirement. One copolymer of the composition (D,L-Ala$_3$, D,L-Orn, L-Leu)$_6$ was actually more active than the natural product. The same lack of specificity appears to be characteristic of at least some antibiotics, as of the "probiotic" strepogenins. However, the excellent synthetic efforts which were invested in obtaining the natural cyclic decapeptide in T. S. Work's laboratory and more recently at Columbia University (304) are not by any means a loss.

Copolymerization of amino acids on to mesoheme IX hydrazids has led to enzyme models with catalytic activities ("phenylene diamine oxidase") not found for the heme or the amino acid polymer alone [Lautsch & Schroeder (305)].

While most amino acid polymerizations are based on the Leuchs' reac-

tion, other reactions have been advocated and successfully employed. A recent addition to this list is the use of N-carbo-thiophenyl amino acids by Noguchi & Hayakawa (306). This reaction made the preparation of copolymers with periodic residue sequences possible.

Of particular interest is the synthesis of the various polyglutamic acids, because of the natural occurrence of such a polymer. Bruckner and coworkers (307, 308) have established that the natural bacterial poly-D-glutamic acid consists largely, if not entirely, of γ-linked residues, and that its properties differ distinctly from the synthetic L- and D-poly-α-glutamic acids. These authors have now succeeded in polymerizing α,α'-dimethyl-γ-L-glutamyl-L-glutamate; the saponified product, believed to be L-γ-polyglutamic acid, resembles the natural polymer in all respects except for a slightly low nitrogen content and the opposite rotation (309). An improved method of saponifying polyamino acid esters was also discovered in Bruckner's laboratory. Apparently the racemizing action of alkali is suppressed, with proteins as well as with polyamino acids, in the presence of cupric hydroxide. This technique has facilitated the preparation of optically active amino acid polymers (310).

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THE HEMOGLOBINS[1]

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Studies up to 1949 on intraspecies differences of hemoglobin were concerned for the most part with the properties of adult and fetal hemoglobin. The monograph by Lemberg & Legge (1) and several chapters of the Barcroft Memorial Volume (2) may be consulted for references to the early work. The discovery in 1949 [Itano & Pauling (3); Pauling *et al.* (4)] of an abnormal hemoglobin in sickle cell anemia stimulated a widespread interest in the study of the hemoglobin molecule in human anemia. Two other abnormal human hemoglobins [Itano & Neel (5); Itano (6)] had been identified when the field was reviewed by Itano in 1953 (7). Of the reviews that have appeared since that time, those by White (8), White & Beaven (9), and Betke (10) place particular emphasis upon the physical and chemical properties of the normal and abnormal hemoglobins. The relationship of the properties and metabolism of the abnormal hemoglobins to disease have been reviewed by Pauling (11, 12) and by Itano (13). Other reviews and monographs have included discussions of the biochemical genetics (14, 15, 16) and the clinical aspects (17 to 20) of the abnormal hemoglobin problem. The present review will place its primary emphasis upon the evaluation of the various physical and chemical criteria on which reports of hemoglobin homogeneity or heterogeneity have been based.

The various hemoglobin compounds will be designated in accordance with the nomenclature proposed by Pauling & Coryell (21), and the word "hemoglobin" will be used as a generic term whenever a more specific designation is not required. The capital letter nomenclature for the molecular species of human hemoglobin is that agreed upon by a group of interested investigators in 1953 (22).

The Human Hemoglobins

The normal adult and fetal hemoglobins of man are designated hemoglobins A and F, respectively. The properties of hemoglobins A and F and of the abnormal hemoglobins S (sickle cell hemoglobin), C, and D have been reviewed by numerous authors. Familial studies to date are consistent with the hypothesis that the synthesis of hemoglobins A, S, C, and D is under the control of allelic genes. The presence of only one of these forms in an individual is believed to be due to homozygosity in the allele controlling its production and the presence of two hemoglobins to the presence of one each of two different alleles. The homozygous states for A, S, and C, corresponding

[1] The survey of literature pertaining to this review was completed in December, 1955.

to the normal state, sickle cell anemia, and to hemoglobin-C disease, respectively, have been observed. Hemoglobin D apparently is extremely rare, only three reports of its occurrence having been added to the original cases [Bird *et al.* (23); Cabannes *et al.* (24); White & Beaven (9)], and homozygosity in the allele for hemoglobin D has not been observed. Hemoglobin F is normally present in infants and is also present in older individuals with chronic anemia. It has been found with A in thalassemia major and in some acquired anemias and with one or two of the abnormal hemoglobins in the abnormal hemoglobin anemias. Specific references to the observations summarized above are available in the reviews cited earlier.

During 1954 and 1955, six more abnormal hemoglobins were reported. The greatly accelerated rate of detection of new forms was in large part due to the successful application of the filter paper electrophoresis technique to the analysis of hemoglobin mixtures (25 to 30). The electrophoretic mobility of hemoglobin E is between those of A and S at pH 6.5 and between those of S and C at pH 8.6 [Itano *et al.* (31); Chernoff *et al.* (32); Sturgeon *et al.* (33)]. With respect to other properties, E does not differ significantly from A (31). E has been found in the homogeneous state [Chernoff *et al.* (34)], as well as in mixtures with A and with F (31, 32, 33), and nearly all occurrences to date have been in Southeast Asia [Graff *et al.* (35); Lie-Injo (36); Na-Nakorn *et al.* (37)]. Hemoglobin G has been observed both in the homogeneous state and in combination with *A* [Edington *et al.* (38, 39)], whereas the only known occurrences of hemoglobins H, I, J, and K are of their mixtures with A [Rigas *et al.* (40); Rucknagel *et al.* (41); Thorup *et al.* (42); Battle & Lewis (43)]. Except for H, the genetic behavior of that of the new forms resembles A, S, C, and D in that each appears to be transmitted from parent to child. Neither parent of the two siblings with H had any abnormal hemoglobin. Further details of the new hemoglobins have been reviewed elsewhere [Itano (13)]. Other reports of inherited abnormalities of human hemoglobin, based on paper electrophoresis above pH 7, have appeared. The migration velocity of the form reported by Schwartz & Spaet (44) resembles that of G in being slightly lower than that of A. The form reported by Cabannes *et al.* (45) resembles I or J in that it migrates faster than A and apparently is transmitted from parent to child. In neither case do the hematologic findings correspond to those reported for earlier occurrences of G, I, or J. Further characterization studies will be necessary to ascertain whether or not the forms reported by Schwartz & Spaet and by Cabannes *et al.*, represent hitherto undescribed species of human hemoglobin. The numerous reports of the heterogeneity of hemoglobin A will be discussed in relation to the method employed in each case.

The Hemoglobins of Others Species

Haurowitz, Hardin & Dicks (46) reported the presence of two components in rat hemoglobin on the basis of kinetic studies of its denaturation in alkali. Using filter paper electrophoresis, Harris & Warren (47) found two

hemoglobins each in goats and sheep, and Cabannes & Serain (48) found two hemoglobins in cattle. The solubility studies of Roche *et al.* (49) on horse and bovine hemoglobin, and of Karvonen & Leppanen (50) on sheep hemoglobin, indicate the presence of more than one component in each. Schapira, Kruh, and co-workers (51 to 55) have reported, on the basis of isotope incorporation studies, the presence of two hemoglobins in rabbits.

Methods

General discussions of the criteria for determining the homogeneity or heterogeneity of proteins have appeared recently (56, 57). The present discussion will be confined to the methods used to demonstrate differences between hemoglobins.

Moving boundary electrophoresis.—The analysis of stroma-free hemolysates of washed red cells in the Tiselius apparatus has been by far the most powerful method for the identification of abnormal hemoglobins. The earliest work on the electrophoresis of human hemoglobin was that of Andersch, Wilson & Menten (58), who reported the resolution of two components in fetal blood in phosphate buffer of pH 7.1; fetal hemoglobin was reported to have a higher anodic mobility than adult hemoglobin. According to more recent studies by Beaven, Hoch & Holiday (59), and by Zinsser (60), adult hemoglobin has the higher anodic mobility in the buffer of Andersch *et al.* Moreover, the mobility difference reported (59, 60) was considerably lower than that reported by Andersch *et al.*; resolution of the fetal and adult hemoglobin boundaries was not obtainable except by use of a special technique [Hoch (61)].

Stern, Reiner & Silber (62) observed two colorless components in addition to hemoglobin in hemolysates from which the stroma had not been removed. No heterogeneity was evident in the hemoglobin component. In addition to the components of the normal human erythrocyte described by Stern *et al.*, Hoch (63) observed two components of greater mobility in 0.01 M Na_2HPO_4 than that of the main oxyhemoglobin component. These components had the absorption spectrum of normal oxyhemoglobin and comprised 1.5 and 2.5 per cent of the total hemoglobin present in the two samples studied. The main component was homogeneous by Hoch's steady state criterion (63).

In the early work on the mobilities of hemoglobins A and S in phosphate buffers, unidentified components were observed with mobilities which corresponded to neither of the major components [Pauling *et al.* (4)]. Studies by Wells & Itano (64) in cacodylate buffer at pH 6.5 revealed the existence of a component with a mobility approximating that of hemoglobin A, in hemoglobin preparations from sickle cell anemia blood; this component was subsequently identified as hemoglobin F [Singer & Fisher (65); Itano (66)]. The proportion of hemoglobin F in sickle cell anemia may be as high as 40 per cent [Itano (67)]. A component with a mobility slightly greater than that of normal adult hemoglobin at pH 6.5 was observed in the hemoglobin of some

normal adults and in individuals with thalassemia minor by Itano and co-workers (68, 69) and by Singer and co-workers (70, 71, 72). Its proportion is low, and its boundary is not well resolved from that of hemoglobin A, so that isolation by the moving boundary method is not feasible. Its identification as hemoglobin S by Singer & Fisher (70) does not seem warranted on the basis of available experimental evidence.

Derrien & Reynaud (73) have reported the appearance of six components in the electrophoresis of hemoglobin A in cacodylate buffer of pH 6.5 and ionic strength 0.1, although in phosphate buffer they observed only two components. Shavitt & Breuer (74), using the same buffer, found only one component at ionic strength 0.1 but observed increasing numbers of boundaries at lower ionic strengths and at elevated temperatures conducive to convection effects. On the other hand, numerous patterns of analyses in cacodylate buffer have been published without any indication of such heterogeneity of hemoglobin-A preparations. In the experience of this author, some commercial preparations of cacodylic acid have produced marked heterogeneity, due perhaps to denaturation or to the binding of impurities. Recrystallization of the cacodylic acid from alcohol has, in each instance, abolished this effect.

The high degree of variation reported by Anderson & Griffiths (75) in the mobilities of hemoglobins A and S has not been observed elsewhere. Reproducible values for absolute mobilites are difficult to attain experimentally because of the numerous variables that must be controlled. Differential mobilities are generally more reliable (61), and the difference between the mobilities of hemoglobins A and S in different sickle cell samples is quite constant. Another observation reported by these workers was that the mobilities of samples taken from the same individual varied from time to time. Although the time interval between samples was not given, it may be pointed out that only 0.8 per cent of a normal individual's hemoglobin is replaced per day. Their suggestion that the observed differences in electrophoretic mobility among the human hemoglobins is due to the reversible complexing of a common hemoglobin with other molecules is not consistent with the results of other studies. Hemoglobins A, S, and C occur naturally in nearly homogenous state, and artificial mixtures separate in essentially the same ratio in which they are prepared, whether the separation procedure is moving boundary electrophoresis [Wells & Itano (64)], filter paper electrophoresis [Motulsky *et al.* (27)], or ion-exchange chromatography [Huisman & Prins (76)]. The virtual absence of phosphate in hemoglobins A and S [Havinga (77)] excludes the possibility of a complex between hemoglobin and nucleic acid. Reversible complexing with small molecules is not involved since the ratio of components in a mixture is not altered by dialysis (64). Electrophoretic studies of the globins from hemoglobins A and S [Havinga & Itano (78)] indicate the presence of an intrinsic difference between respective globins which survives the treatment involved in their preparation.

All of the known human hemoglobins have been examined as carbon-

monoxyhemoglobin by moving boundary electrophoresis.[2] Hemoglobin H is isoelectric, and the other forms carry positive charges in cacodylate buffer of pH 6.2. In cacodylate buffer of pH 6.5, hemoglobin H is a negative ion and the others are positive. Hemoglobin C has the highest mobility at pH 6.5, and the relative mobilities are as follows: C > S = D ≧ G > E > A > F > J > I. In barbital buffer of pH 8.6 all of the forms are negatively charged, and their relative mobilities are as follows: H > I > J > A > F > G > S = D > E > C. The ionic strength of each of the buffers is 0.1.

Because the pH-mobility curves of some of the hemoglobins do not parallel each other, the pH for optimum separation varies with different mixtures. In some cases the difference in mobility is so small that separation of the boundaries may be obscured by diffusion. Hoch (59) used 0.01 *M* Na_2HPO_4 to separate hemoglobins A and F, which are not resolvable in buffers of ionic strength 0.1. Morrison *et al.* (79) compared a variety of buffers and found that mixtures of A, S, F, and C were best separated in barbital buffer of pH 8.8 and ionic strength 0.06. Van der Schaaf & Huisman (80) separated A, S, and F in phosphate buffer of pH 6.8 and ionic strength 0.01. The separation with use of dilute buffers is frequently much sharper in one limb than in the other; moreover, corrections must be applied to the apparent mobilities and compositions to obtain true values. Hoch's procedure, for example, results in sharpened ascending boundaries and in an apparent increase in the proportion of the faster component (59, 63). References to the literature on these theoretical considerations are available in a review by Longsworth (81).

Zone electrophoresis.—A number of modifications of the filter paper electrophoresis method have been used with success for the detection of abnormal human hemoglobins (25 to 30). Hemoglobin is especially well suited to this method since its migration can be observed without the use of protein stains. The relative proportions in a mixture can be determined by elution [Larson & Ranney (26)] or by direct photometric scanning [Motulsky, Paul & Durrum (27)] of the paper strip; good approximations are obtainable from direct visual estimates. In the usual case, oxyhemoglobin solutions prepared from washed cells are analyzed, although hemolysates from the whole blood have also been used. Tuttle (82) has shown that a substance present in normal plasma may interact with hemoglobin and alter the migration behavior of the latter on paper.

The usual buffer employed in filter paper electrophoresis is barbital at

[2] Itano, H. A., unpublished studies. The author is indebted to the investigators who furnished samples of the abnormal hemoglobins. Edington *et al.* (39) reported that at pH 6.5 the mobility of G is slightly less than that of S. In our experiments no difference was found at pH 6.5, but at pH 8.6 the difference between S and G was easily demonstrable. There was, however, some denaturation in the sample received by the author. The resolution of A and K at pH 6.5, reported by Battle & Lewis (43), was not obtained by the author in samples from the original subjects. On the other hand, a small component corresponding to the rapid component previously observed in normal and thalassemia minor subjects (68 to 72) was present.

pH 8.6 and ionic strength about 0.05. The reliance of most investigators on this buffer alone is undesirable in that certain hemoglobins migrate together at one pH and separate well at another. For example, S and F, which are poorly resolved at pH 8.6, are well resolved at pH 6.5. S and E, on the other hand, separate better at pH 8.6 than at pH 6.5 (31, 33). In practice, considerably more denaturation takes place on paper at pH 6.5 than at 8.6, and the oxidation of hemoglobin to ferrihemoglobin takes place more rapidly in acidic than in alkaline media. Nevertheless, the use of acidic buffers has proven a valuable adjunct to alkaline buffer electrophoresis on filter paper.[3]

Small components which form detectable boundaries in the Tiselius apparatus may go undetected on paper due to adsorption and to spreading of the zone of the main component. However, zone electrophoresis offers a distinct advantage in some cases when the complete isolation of a component is desired. By increasing the capacity of the supporting medium with the use of starch slabs, Kunkel & Wallenius (83) were able to analyze a larger amount of hemoglobin than is permitted with paper. A small component having the mobility of hemoglobin E was separated from the hemoglobin samples from normal subjects and from individuals with thalassemia minor. It is likely that this component is the same as that previously observed at pH 6.5 (68 to 72) in similar samples by the moving boundary method. It differs from the components separated by Hoch (63) at high pH in that it migrates more slowly than hemoglobin A.

Kolin (84) has attained extremely rapid electrophoretic separation of protein mixtures by the simultaneous use of pH and density gradients. Each component separates out in a narrow zone at the pH of its isoelectric point. For use in the identification of abnormal hemoglobins a scheme for measuring the pH of these zones will be necessary. The addition of known hemoglobins to the unknown mixture may serve this purpose. Hoch & Barr (85) conducted filter paper electrophoresis with a superimposed pH gradient and obtained good separation of the electrophoretic components of serum proteins but not of hemoglobins A and F, which differ very little in their isoelectric points.

Solubility determinations.—The presence of inhomogeneity in protein samples may be detected by the application of the phase rule to solubility determinations at constant salt concentration. By this method the hemoglobin of normal human adults behaves as a single component, both as crystalline CO-hemoglobin and as amorphous ferrohemoglobin [Jope & O'Brien (86); Itano (67)], while the CO-hemoglobin of the horse behaves as a mixture [Roche *et al.* (49)]. The mixture of hemoglobins A and S present in sickle cell trait behaves as a solid solution (67). In general, mixtures of the human hemoglobins apparently form solid solutions with solubilities in phosphate that lie between those of the component species although mixtures of hemoglobins A and F may be exceptional. The solubility of

[3] Bergren, W. R. (Personal communication to the author)

crystalline adult ferrohemoglobin in phosphate is about twice that of fetal (86), but the solubilities of mixtures of amorphous ferrohemoglobins A and F are about 50 per cent greater than that of A (67). These observations suggest that hemoglobins A and F form mixtures with additive solubilities instead of solid solutions with intermediate solubilities and are consistent with the observations that A and F do not form isomorphous crystals [Jope & O'Brien (86); Bragg & Perutz (87); Perutz *et al.* (88); Zinsser & Tang (89)]. Another possible explanation is that the relative solubilities of A and F differ in their crystalline and amorphous modifications.

On the basis of salting-out experiments, Roche, Derrien, and co-workers (49, 90, 91, 92) have reported heterogeneity in a number of different hemoglobins. Their results on hemoglobin A, which suggested the presence of three components, could not be confirmed by Jope & O'Brien (86), whose results by the same method did not suggest the presence of more than one component. The discontinuities in salting-out curves, on which the conclusions of Roche *et al.* are based, probably indicate changes in the nature of the solid phase, and such changes may result either from the precipitation of a different form of hemoglobin or from a change in the type of aggregation of the same hemoglobin due to interaction with small molecules or ions in the solvent. Hemoglobins A, S, and F, as well as the hemoglobins of other species, are known to crystallize in more than one form (86 to 89, 93), depending on the composition of the solvent. It seems unlikely that each of the crystalline forms of a given hemoglobin would exhibit salting-out curves with the same slope, and the discontinuities may represent transition points between the regions of stability of two solid phases. In this connection, it is of interest that Karvonen & Leppanen (50) found a critical pH at which two types of crystals of sheep hemoglobin precipitated simultaneously. One of the forms precipitated alone at higher pH and the other at lower pH.

Roche *et al.* (49) achieved a partial fractionation of their sample of horse hemoglobin by their salting-out procedure. They also reported a successful fractionation of bovine hemoglobin. However, in terms of their interpretation of the salting-out plot, anomalous results were obtained with the fetal hemoglobin of infants and of sickle cell anemia patients [Roche *et al.* (94); Derrien *et al.* (95, 96)]. Thus, it has not been established unequivocally that discontinuities in the salting-out plot always indicate heterogeneity of a protein. The number of discontinuities seems always to exceed the number of components detectable by other methods. The application of this method to the estimation of proportions of components in a hemoglobin mixture (95, 96) has no theoretical basis.

Perutz *et al.* (88, 97) discovered that the solubility of ferrohemoglobin S in phosphate buffer is a small fraction of that of ferrohemoglobin A, although the solubilities of the oxy- and ferri-forms of A and S are nearly equal. The solubility of amorphous ferrohemoglobin C in phosphate buffer of pH 6.8 is higher than that of A [Itano (67)]. Thomas *et al.* (98) also observed a high solubility for ferrohemoglobin C at pH 6.8, but Huisman *et al.*

(99), who used phosphate buffer of pH 6.4, reported that ferrohemoglobin C has a lower solubility than A. Conflicting reports have also appeared on the solubility of CO-hemoglobin C. Derrien *et al.* (92) observed abnormally high solubilities in phosphate buffers of pH 6.4, and Huisman *et al.* (99), using the same method, observed low solubilities. Perhaps related to these results is the observation of intraerythrocytic hemoglobin crystals in one case of apparent hemoglobin C disease by Diggs *et al.* (100). None of the other cases of hemoglobin C disease reported to date have had these crystals. The possibility thus exists that there are two different forms of hemoglobin having the electrophoretic mobility of hemoglobin C but differing in solubility.

Denaturation experiments.—The resistance of hemoglobin F to denaturation by alkali is much greater than that of the other human hemoglobins, and the reaction is used both to identify hemoglobin F and to estimate its proportion in a mixture. The denaturation process may be followed by observing the change in optical density of the homogeneous reaction mixture or by quenching the reaction by neutralization and measuring the concentration of undenatured hemoglobin remaining in solution. The reaction is first-order with respect to the hemoglobin molecule in the case of a number of species, so it has been assumed that deviations from this mechanism indicate inhomogeneity.

The apparent course of the alkali denaturation reaction differs when studied by the precipitation method and when followed optically in homogeneous solution, and conflicting observations have been reported. Although White & Beaven (9) have reported that hemoglobin A denatures according to first-order kinetics by the photometric method, other investigators have reported a more complex mechanism [Jonxis (101); Betke (102)]. White & Beaven also found that the alkali denaturation of oxyhemoglobin F obeys first-order kinetics by the photometric method, but that CO-hemoglobin F does not. According to Betke (103), oxyhemoglobin A does not follow first-order kinetics by either method, and CO-hemoglobin A follows this mechanism by the photometric method but not by the precipitation method. There appears to be general agreement that the apparent course of oxyhemoglobin F denaturation follows first-order kinetics by either method [White & Beaven (9); Jonxis (101); Betke (102); Singer *et al.* (104)], but the two methods do not yield the same result for the apparent proportion of F [van der Schaaf & Huisman (80)].

The final reaction product of alkali denaturation in the presence of oxygen is alkaline globin ferrihemochromogen. The contribution of globin ferrohemochromogen and other possible denaturation intermediates [Jope (105)] has not been analyzed. When the precipitation method is used, errors may occur from the measurable solubility of denatured hemoglobin in the precipitating salt solution and to the coprecipitation of undenatured material with the denatured. Moreover, it is improbable that the conversion of a hemoglobin molecule to an insoluble product and the change of the

absorption spectrum associated with all four of its heme groups to that of alkaline globin ferrihemoglobin take place simultaneously. Neglect of these factors would produce a large relative error in the analysis of mixtures containing a small proportion of hemoglobin F and may account for the wide divergence observed by Singer *et al.* (104) in the calculated denaturation rate of the F component in sickle cell anemia hemoglobin. The difference in the alkali denaturation rates of hemoglobins A and F is such that with a suitable choice of conditions nearly all of the F in a mixture remains in solution when all of the A is denatured. However, hemoglobin F prepared in this manner cannot be made to crystallize [Chernoff (106)], an observation which suggests that exposure to alkali results in alterations in molecular configuration without producing insolubility.

When the alkali denaturation of oxyhemoglobin A does not follow the simple first-order law, the plot of the course of the reaction may be fitted by a function which is the sum of two exponential terms (102). These results have long been interpreted as being indicative of the presence of a rapidly denaturing and a slowly denaturing fraction in hemoglobin A. If this were the case, partial denaturation of a sample and removal of the denatured portion would result in a relative increase of the more resistant component in the undenatured portion. Betke (107) carried out this test and found that the undenatured portion, when exposed to alkali, followed the same reaction course by the photometric method as the original sample, so that no apparent fractionation had occurred. When the test was repeated with use of the precipitation method, the reaction curve of the product of partial denaturation fell between those of the original sample and of a curve calculated on the assumption that partial fractionation of a two-component mixture had occurred [Betke (10)]. Betke concluded that either the reaction is not first-order or that there exist different states of hemoglobin that are not sharply defined and are interconvertible. A third alternative is that a hemoglobin sample may be homogeneous but that its conversion from oxyhemoglobin to globin ferrihemochromogen proceeds in a series of reactions. There is only one rate-determining step in the alkali denaturation of hemoglobin F, but at least two are observable with A. The confused situation with respect to the alkali denaturation reaction points out the necessity of a more critical analysis of the data and of the assumptions heretofore taken for granted.

Chromatographic methods.—The separation of adult hemoglobin into several components by chromatographic methods has been claimed by several investigators (108 to 113). To date the products of the apparent separations have not been closely scrutinized by physical and chemical methods to determine whether they represent unaltered fractions of the original sample. Morrison & Cook (110) have reported the separation, on an IRC-50 column, of normal adult oxyhemoglobin into three native components; the absorption spectra of the components were reported to be normal. On the other hand, Jope (105) has shown that partially denatured oxyhemoglobin molecules have normal visible spectra. According to an earlier

communication by Morrison & Cook (111), one of the components contains an altered porphyrin. Another component could be eluted off their IRC-50 column after the major component.

A similar inhomogeneity was noted by Boardman & Partridge (114) in their work with sheep and bovine CO-hemoglobin on the same resin. When the earliest eluting component was passed through the same column, the more slowly eluting component again appeared. The absorption spectrum of the latter was reported to be similar to that of ferrihemoglobin. The relative amount of the second component was markedly reduced when the experiment was carried out at low temperature.

Huisman & Prins (76, 115) have separated CO-hemoglobins A, F, S, and C, also on IRC-50. Essentially complete recoveries of hemoglobins A, S, F, and C were obtained at 0°C. At 10° the recovery of hemoglobins A and C was diminished to 81 and 72.5 per cent, respectively. At 20°C., when a mixture of hemoglobins A and F was chromatographed, a third band ascribed by the authors to ferrihemoglobin appeared.

Sedimentation and diffusion studies.—According to Taylor & Swarm (116), the sedimentation constants of hemoglobins A and F are identical. Their diffusion constant value (D_{20}) of 6.6×10^{-7}cm.^{2} sec.$^{-1}$ for F is similar to reported values for A, and indicates that the two have similar molecular weights. Andersch *et al.* (58) had earlier reported that hemoglobin F has a lower s_{20} than that of adult hemoglobin and had suggested that the molecular weight of F was half that of A. Kegeles & Gutter (117) have reported a s_{20} value of 4.17 S and Field & O'Brien (118) a value of 4.24 S for carbonmonoxyhemoglobin A at one per cent concentration.

The sedimentation and diffusion studies of Field & O'Brien indicate that hemoglobin A is reversibly dissociable between pH 3.5 and 6.0 and that it is stable between pH 6.0 and 11. The value of 6.9×10^{-7} cm.^{2} sec.$^{-1}$ obtained by Field & O'Brien for the diffusion constant of hemoglobin A is the same as that reported earlier by Lamm & Polson (119), and is significantly higher than the value of 6.3×10^{-7} found by Tiselius & Gross (120) for horse hemoglobin. The dissimilarity in diffusion constants stands in contrast to the similarity in both the external and internal structure of horse and human hemoglobin molecules [Bragg & Perutz (87); Perutz, *et al.* (88)].

Calculations based on velocity sedimentation and diffusion have yielded low values for the molecular weight of undissociated human hemoglobin. The latest value of 59,600 reported by Field & O'Brien is significantly lower than values based on osmotic pressure and iron content determinations. The iron content of crystalline hemoglobin A, 0.338 to 0.340 per cent (121, 122), corresponds to a molecular weight of 66,000 for an undissociated molecule containing four iron atoms. Recent studies[4] have yielded lower values for the diffusion constant of hemoglobin A than those previously reported.

[4] Gutter, F. J., Sober, H. A., and Peterson, E. A., *Arch. Biochem. and Biophys.* (In press)

Amino acid analyses.—Several workers have found five N-terminal valines in adult hemoglobin [Porter & Sanger (123); Brown (124); Schapira & Dreyfus (125)]. Havinga (77) found four or five terminal valine residues per molecule for both normal adult and sickle cell hemoglobin, and Masri & Singer (126) have reported four terminal valines for hemoglobins A, S, and C. Amino acid analyses of different forms of human hemoglobin have been published by several groups (127 to 131). Differences between normal adult and sickle cell hemoglobins are small although minor differences have been reported in the uncharged amino acids. The most striking difference reported in hemoglobin C is its high content of lysine [Huisman *et al.* (129)]. Fetal hemoglobin has a significantly different amino acid composition from the others, a notable difference being its high isoleucine content [Dustin *et al.* (128); Huisman *et al.* (129, 130)]. The amide nitrogen contents of hemoglobins A and S do not differ significantly [Schroeder *et al.* (127); Dickman & Moncrief (132)]. According to filter paper electrophoresis studies by Scheinberg *et al.* (133), hemoglobins A, S, and C differ in their content of ionizable carboxyl groups. There is a lack of correspondence between their results and the amino acid compositions of the hemoglobins, especially with respect to hemoglobins A and C.

Sulfhydryl groups.—Ingbar & Kass (134) titrated hemoglobins A and S with silver nitrate in ammoniacal solution and found two and three SH groups per molecule, respectively. The addition of 20 per cent methanol increased the number of titratable groups to four to five for each. Using silver nitrate in more dilute ammonia, Ingram (135) found four SH groups in native hemoglobin A. He suggested that the discrepancy between his results and those of Ingbar & Kass might be due to the higher concentration of ammonia used by the latter workers.

The observations of Ingram on the binding of *p*-chloromercuribenzoic acid and methylmercury nitrate by native hemoglobin A are in accord with an earlier report by Hughes to the effect (136) that a molecule of native hemoglobin A reacts with two molecules of methylmercury iodide. Both Ingram (135) and Benesch *et al.* (137) found eight SH groups in denatured hemoglobin A. The sulfhydryl contents of horse, ox, and sheep hemoglobins were also determined by Ingram, and that of sheep and dog hemoglobin by Benesch *et al.*

Oxygen equilibrium studies.—Allen & Wyman (138) observed no difference between the oxygen equilibria of hemoglobins A and S, either in dilute solution or in concentrated solutions in which ferrohemoglobin S aggregates into tactoids [Harris (139)]. Becklake *et al.* (140) reported that whole blood from sickle cell anemia patients has a decreased affinity for oxygen, while whole blood from sickle cell trait subjects behaves normally. The discrepancy between observations on whole blood and on hemoglobin solutions also applies to hemoglobins A and F [Allen *et al.* (141)]. According to several earlier studies of fetal blood or washed erythrocytes, hemoglobin F has a greater affinity for oxygen than the corresponding maternal specimen. Allen *et al.*,

working with hemoglobin solutions, observed that with undialyzed samples from freshly laked cells maternal hemoglobin had a greater oxygen affinity than fetal. When the hemoglobin samples were dialyzed simultaneously against a common surrounding medium, the oxygen affinities of fetal and maternal hemoglobin became equal. The extent of interaction between the hemes was the same for fetal and maternal blood and remained unchanged with dialysis. (The experiments of Allen *et al.* were carried out at pH 7.0 to 7.4.)

AT pH 8.6, Riggs (142) observed a decrease in heme interaction after dialysis and a partial reversal of this effect upon the addition of glutathione. The addition of the sulfhydryl blocking agent, *p*-chloromercuribenzoate, resulted in a marked decrease in heme-heme interaction, partially reversible by the addition of glutathione. Using horse hemoglobin, Wolbach & Riggs (143) have conducted further studies on the effect of sulfhydryl blocking reagents on oxygen affinity and heme interactions. According to Guthe (144), the addition of formaldehyde to the equilibrium system results in a considerable increase of the oxygen affinity and decrease of the heme interaction of human hemoglobin. These studies emphasize the need for careful preparation of samples and control of conditions in comparative studies of the oxygen equilibria of different hemoglobins.

Absorption spectra.—Jope (105) has shown that in human fetal hemoglobin the tryptophan absorption band appears at 289.8 mμ; in human adult hemoglobin and in the adult and fetal hemoglobins of other mammalian species, the position of the band is 291.0 mμ. Betke (10), Lambrechts & Martin (145), Beaven *et al.* (59), and Greinacher *et al.* (146) compared hemoglobins A and F in the visible and infrared regions, and observed no significant differences.

With one exception, no other spectral differences have been reported among the human hemoglobins. Hörlein & Weber (147) reported cases of congenital ferrihemoglobinemia in which the acid ferrihemoglobin had a visible absorption spectrum which was significantly abnormal. The spectrum was not altered by replacement of the hemes with heme from normal hemoglobin, so it was concluded that the abnormality resided in the globin. Alkaline ferrihemoglobin and the compounds of ferrihemoglobin with fluoride, cyanate, and cyanide were reportedly normal. The findings of Hörlein & Weber on acid ferrihemoglobin are remarkable in view of the fact that the hemoglobins of different species, though differing in their globin composition, have visible absorption spectra which are nearly identical. It is not evident whether the apparent abnormality is due to an inherent defect of the hemoglobin molecule or to a degradative change caused by an inherited metabolic defect not related to hemoglobin synthesis.

Immunologic studies.—Goodman & Campbell (148), Chernoff (106), and Aksoy (149), using rabbit sera, found significant differences in antigenic specificities between hemoglobins A and F but not between A and S. Using chicken sera in precipitation reactions carried out at high salt concentration, Goodman & Campbell (148) were able to obtain discernible differences between the specificities of hemoglobins A and S, but their data suggested a

great similarity in the antigenic groups of the two hemoglobins. The alkali resistant fraction of sickle cell anemia hemoglobin and those of hemoglobins from other chronic anemias were found to have the antigenic specificity of hemoglobin F.

With the ring test technique, Chernoff (106) detected material which reacted with anti-F sera in the hemoglobin of normal subjects in amounts as low as 0.03 per cent of the total hemoglobin present. The sensitivity of the ring test is so great that the possibility that the reaction resulted from the presence of non-hemoglobin impurities in both the immunizing and test antigens cannot be ignored. The comparison of the ring test results with the results of the alkali denaturation technique of Singer *et al.* (104) is not valid at low concentrations, as the soluble residue of 0.5 to 2 per cent of the total hemoglobin obtained from all non-anemic hemoglobins by the denaturation method contains little, if any, fetal hemoglobin.

Studies of Hemoglobin Metabolism

Schapira, Kruh, and co-workers (51 to 55) have carried out a series of investigations on the incorporation of Fe^{59} by rabbit hemoglobin. Following the injection of Fe^{59} they separated the hemoglobin in rabbits into two fractions by a variety of physical and chemical methods. In each case, the two fractions were unequally labeled. If, as Schapira and his co-workers concluded, there exists in the rabbit two forms of hemoglobin, one of which is synthesized more rapidly than the other, the relative rates of synthesis should be equal to the proportions of the two forms in the peripheral blood. Dissimilarity between the two ratios would indicate either that two forms are produced in different cells with different survival times, or that one form is transformed into the other. As stated by the authors, the fractionations were performed arbitrarily, so that the fractions obtained could not be identified as homogeneous entities. The proportions of the two fractions obtained and the ratios of their specific activities depended on the method of fractionation employed. Their data, therefore, do not permit a choice between their hypothesis and the alternatives given above. In fact, the arbitrary limitation of the number of forms present in rabbit hemoglobin to two may not be justified.

When Schapira *et al.* (51) administered Fe^{59} to anemic rabbits, they observed a greater difference in the specific activities of hemoglobin fractions obtained by alkali denaturation than they had observed in normal rabbits. In each group, the alkali resistant fraction had the greater specific activity. Benard *et al.* (150) found that in rabbits recovering from artificially induced anemia, the newly formed hemoglobin was of the alkali-resistant variety. As the erythrocyte count approached pre-anemic values, the proportion of alkali-labile hemoglobin also approached pre-anemic values. Their observations suggest that rabbit hemoglobin, when initially formed, is alkali-resistant and that it becomes alkali-labile with time.

From electrophoretic data, which show that there is more hemoglobin A than S or C in individuals heterozygous for the abnormal hemoglobin genes,

Itano (13, 151) has inferred that the genetically controlled mechanism for the synthesis of S or C is less efficient than that for A. According to this hypothesis, homozygosity in either gene would result in a subnormal rate of hemoglobin synthesis. While this hypothesis is difficult to test on a cellular basis, erythrocyte survival and isotope incorporation studies indicate the existence of a relative inhibition of total hemoglobin production, both in sickle cell anemia (homozygosity for S) and in hemoglobin C disease (homozygosity for C) [Crosby & Akeroyd (152); Thomas *et al.* (98); Terry *et al.* (153); James & Abbott (154)].

Fetal hemoglobin is produced in every individual during prenatal and early postnatal life, and beyond infancy in some individuals with chronic anemia. Studies by Walker & Turnbull (155) suggest that anoxia stimulates fetal hemoglobin production in the fetus. Fetuses that were anoxic prior to delivery, because of various clinical abnormalities, had high hemoglobin concentrations. Comparison of their blood with that of normal infants revealed that the increase could be ascribed to an increased production of fetal hemoglobin. Perhaps the basic stimulus for fetal hemoglobin production in chronic anemia is also anoxia.

Dreyfus *et al.* (156) separated the adult and fetal hemoglobins of cord blood after incubation of the blood with Fe^{59}. They found that the specific activity of the adult form was twice as great as that of the fetal, and concluded that the adult fraction incorporated iron more rapidly than did the fetal. However, since on the average there is about three times as much fetal hemoglobin as adult in cord blood, the total activity of the fetal fraction was about one and a half times that of the adult. It is more likely that the increased rate of iron incorporation in cord blood is related to its reticulocytosis, and that the total incorporation ratio corresponds to the relative rates of synthesis of the two hemoglobins in the human at the time of birth.

Drescher & Künzer (157) observed that hemoglobin, from fetuses of 7 to 12 weeks' gestation, denatured in alkali at a rate intermediate to the denaturation rates of fetal and adult hemoglobins. They ascribed this behavior to the presence of a third normal type of hemoglobin which is produced only in early fetal life. Walker & Turnbull (155), on the other hand, found only fetal hemoglobin in the eleventh and twelfth weeks, and observed small amounts of adult hemoglobin by the thirteenth week.

The available genetic and hematologic evidence has indicated that hemoglobins S and C are abnormal forms of adult hemoglobin (7). Recent studies of infant blood by paper electrophoresis have shown that these forms are undetectable or are present in small amounts at birth, and that their proportion increases with time [Terry *et al.* (153); Schneider & Haggard (158)]. The proportion of fetal hemoglobin has been observed to be as high as 20 per cent in blood in which all or nearly all of the cells are capable of sickling [Neel *et al.* (69); Schneider & Haggard (158)], so that hemoglobins S and F must co-exist in at least a portion of the cells. No direct evidence is available as to whether or not hemoglobins A and F occur together in the same cell.

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METABOLISM OF AMINO ACIDS AND PROTEINS

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The subjects of amino acids and of proteins, which were written by the respective coauthors, will be presented separately, each with its own bibliography.

I. METABOLISM OF AMINO ACIDS[1]

By Edward A. Adelberg

Few fields have been so frequently and so comprehensively reviewed as amino acid metabolism. The reader who wants the complete picture may begin with Volume II of *Chemical Pathways of Metabolism*, edited by Greenberg in 1954 (1), and then assimilate *Amino Acid Metabolism*, which in 1048 pages summarizes the symposium sponsored by the McCollum Pratt Institute in June, 1954 (2). For those who wish to specialize, there are reviews on amino acid biosynthesis [Davis (3)], transamination [Meister (4)], and on glutamine metabolism [Meister (5)]; the amino acid symposium of the American Society of Biological Chemists held in April, 1955 [transamination, microbial metabolism, hormonal influences, and regulatory interrelations with carbohydrate metabolism (6)]; and, in the present volume, a review of transaminases and racemases [Meister (7)]. In case some detail is missed in the above, the masterly review by Ehrensvärd in last year's *Annual Review of Biochemistry* (8) will undoubtedly fill in the gaps.

In the face of such intensive coverage, the present reviewer has felt it unnecessary to give a comprehensive picture of the metabolism of each group of amino acids, and instead has tried to pick up the loose ends and bring the field up to date as of November, 1955.

Unsubstituted, Unbranched, Aliphatic Amino Acids

Functions of alanine.—The role of L-alanine in the germination of *Bacillus* spores has been studied by Harrell & Halvorson (9). Their elegant methods for controlling germination have enabled them to show that alanine

[1] The following abbreviations are used in this section: ADP for adenosinediphosphate; AEC for S-aminoethylcysteine; ATP for adenosinetriphosphate; CAP for carbamylphosphate; DAP for diaminopimelic acid; DPN for diphosphopyridine nucleotide; IGP for imidazoleglycerolphosphate; ITP for inosinetriphosphate; 3-PGA for 3-phosphoglyceric acid; SDP for sedoheptulosediphosphate; THFA for tetrahydrofolic acid.

is actively bound during a 45-sec. exposure of the spores to this amino acid, which exposure is sufficient to trigger the subsequent germination of 40 per cent of the spores. However, no net metabolism of L-alanine was detectable during germination. Alanine racemase is uniquely active in such spores (10). Thorne and his co-workers have found that this enzyme, by coupling with two stereospecific transaminases, brings about the racemization of glutamic acid in *Bacillus* species (11, 12). Perhaps a similar indirect function for alanine exists in the germination process.

Oxidative catabolism.—The breakdown of C^{14}-β-alanine in the intact rat was reported to result in the formation of carbon dioxide and C_2 fragments [Pihl & Fritzson (13)]. An oxidative pathway involving formylacetic acid as an intermediate was proposed. Kinnory *et al.* have traced the catabolism of C^{14}-labeled α-aminobutyrate, norvaline, and norleucine in rat liver homogenates, and find that in every case the amino acid is first converted to the corresponding α-keto acid, followed by oxidative decarboxylation to the next lower monocarboxylic acid, and that the latter product is then oxidized by the classical pathway of β-oxidation (14,15).

Glycine and Serine

Biosynthesis and interconversion.—The evidence continues to mount that serine arises first in the *de novo* synthesis of these amino acids. Ichihara & Greenberg (16) have now established the synthesis of serine from 3-phosphoglyceric acid (3-PGA) via 3-phosphohydroxypyruvate. Their enzyme preparations from rat liver synthesize serine from the latter intermediate by reactions which include transamination from alanine as one step. Their published experiments left undecided the following alternative paths:

phosphohydroxypyruvate → hydroxypyruvate → serine **1.**

phosphohydroxypyruvate → phosphoserine → serine **2.**

More recently (17) they have demonstrated the formation of phosphoserine from 3-PGA by their preparations. The formation of phosphoserine from serine itself was ruled out by carrying out the reaction in the presence of C^{14}-serine; the phosphoserine which was isolated was not labeled. Furthermore, their preparations were found to contain two phosphatases active on phosphoserine, but no serine kinase. The pathway shown in reaction 2 thus seems fairly well established.

This finding explains the many earlier reports on the metabolic conversion of labeled pyruvate to differently labeled serine. For example, Nyc & Zabin (18) found that pyruvate-3-C^{14} injected into rats yielded serine labeled equally in carbons 2 and 3, which suggested passage through a symmetrical C_4 intermediate. It has now been shown that oxalacetate, which is formed directly from pyruvate and equilibrates with symmetrical compounds in many animal tissues, can react with ATP (19) or ITP (20) to form phosphoenolpyruvate plus carbon dioxide. Bartley & Avi-Dor (21) showed that longer incubations result in formation of 3-PGA. The following

sequence is thus involved in the conversion of pyruvate to serine: pyruvate-3-C^{14} + CO_2 → oxalacetate-3-C^{14} ⇌ oxalacetate-2,3-C^{14} → phosphoenolpyruvate-2-3,-C^{14} → 3-PGA-2,3-C^{14} → serine-2,3-C^{14}.

The synthesis of serine is followed in all organisms by a rapid, reversible conversion to glycine. This appears to be the major source of glycine in animal tissues and in microorganisms. Glycine can also be formed by the transamination of glyoxylate, which can arise in microorganisms by cleavage of isocitrate (22); however, isotopic competition experiments in *Escherichia coli* (23, 24), *Neurospora crassa*, and yeast (25) indicate that this path plays a quantitatively minor role. A small amount of glycine may also be formed by cleavage of exogenously furnished threonine, both in mammals (26; see also review, 3) and in *E. coli* (27). In the latter case, Roberts has cited good evidence that threonine synthesized endogenously is not converted to glycine.

The mechanism of the interconversion of serine and glycine has been the subject of many reports. Alexander & Greenberg (28), Blakeley (29), and Kisliuk & Sakami (30) have independently concluded that in animal tissues tetrahydrofolic acid (THFA) is the true C_1 carrier, and that related folic acid derivatives act only after conversion to THFA. There is also general agreement that the C_1 unit is carried at the oxidation level of formaldehyde, and both hydroxymethyl-THFA (28, 30) and a cyclized form of hydroxymethyl-THFA (29) have been proposed. However, Jaenicke (31) has prepared N(10)-formyl-THFA, both enzymatically and by catalytic hydrogenation of N(10)-formylfolic acid, and has shown that the first compound formed by the transfer of the serine β-carbon to THFA is an N(10) derivative. Citrovorum factor (CF, N(5)-formyl-THFA) is apparently a by-product. N(10)-formyl-THFA was also shown to act as a C_1-donor for serine, purine, and histidine synthesis. In the glycine-serine conversion, N(10)-hydroxymethyl-THFA seems to be involved; it can be reversibly oxidized with DPN to N(10)-formyl-THFA. Greenberg, Jaenicke & Silverman (32) have studied the enzymatic formylation of THFA by formate plus ATP and have established that N(10)-formyl-THFA directly transformylates 5-amino-4-imidazolecarboxamide-5′-phosphoriboside in the synthesis of the purine ring.

Wright (33, 34) has discovered a new form of the coenzyme, closely related to a tri-glutamyl type of leucovorin (N(5)-formyl-THFA), required for the serine-to-glycine conversion in extracts of *Clostridium* sp. The new coenzyme, called "Co C," isextra ctable from boiled extracts of *Clostridium cylindrosporum.*

Conversion of serine and glycine to other cell constituents.—Serine is a precursor of several amino acids (see 3): tryptophan, glycine, and cysteine. Serine can also act as a C_1 donor to the folic acid system, by means of which the β-carbon of serine can be used in the synthesis of thymine, methionine, and purine (see 30). That serine may furnish a portion of the carbon skeleton of riboflavin has been deduced from its stimulatory effect on riboflavin

production by *Eremothecium ashbyii* (35, 36), but tracer confirmation is needed. The decarboxylation of serine also furnishes the ethanolamine moiety of choline (see 37); ethanolamine in turn may possibly be a precursor of the mercaptoethylamine moiety of coenzyme A (see discussion of sulfur-containing amino acids, below).

Glycine is an important building block for porphyrins (38, 39) via succinylglycine, the metabolism of which can also result in the transfer of the α-carbon of glycine to the folic acid system (40). The α-carbon of glycine was found to be incorporated into β-carotene by *Phycomyces blakesleeanus*, and δ-aminolevulinic acid (a product of succinylglycine metabolism) was then shown to stimulate carotene production in this organism [Mackinney *et al.* (41)]. However, the authors cautiously refrain from speculation concerning the mechanism.

The mechanism by which glycine is used as a building block for purine synthesis has been beautifully elucidated by Goldthwait, Greenberg & Peabody (42, 43) and by Levenberg, Buchanan and co-workers (44, 45, 46). The first step is the conversion of glycine to glycinamide ribotide by a reaction involving 5-phosphoribosylamine plus ATP.

Metabolism of serine and glycine peptides.—Meinhart & Simmonds (47) carried out an extensive study of the nutritional activity of certain peptides for serine-glycine auxotrophs of *E. coli*. All the peptides tried appeared to be used after hydrolysis to free amino acids. The use of L-serylglycine by a serine-requiring mutant was anomalous; although this mutant contains a peptidase for serylglycine and does not metabolize free glycine during growth no free glycine accumulated during growth on serylglycine. Serylglycine was used for growth more efficiently than free serine by this mutant, meaning either that the serine residue in this peptide is relatively protected from catabolic attack, or that it can be used for protein synthesis by a different route from that by which free serine is used.

Naturally-occurring derivatives of serine.—An impressive array of serine derivatives occurs naturally. To phosphoserine from animal sources (e.g., 48) can now be added the following of microbial origin: two antibiotics, azaserine (I) (49) and cycloserine (50) which is also called oxamycin (II) (51, 52); a nonantibiotic product of a *Streptomyces*,—O-carbamyl-D-serine (III) (53); and serratamic acid,—a peptide of L-serine and a hydroxydecanoic acid found in a species of *Serratia* (54).

$$N^+{=}N^-{=}CH\cdot CO\cdot O\cdot CH_2\cdot CH(NH_2)\cdot COOH \qquad \overbrace{O\cdot CH_2\cdot CH(NH_2)\cdot CO}^{NH}$$

I Azaserine   II Cycloserine (oxamycin)

$$H_2N\cdot CO\cdot O\cdot CH_2\cdot CH(NH_2)\cdot COOH$$

III O-Carbamyl-D-Serine

Azaserine, which is antineoplastic (49) and mutagenic (55) has been

found by Buchanan and co-workers (46) specifically to antagonize glutamine in its enzymatic reaction with formylglycinamide ribotide and ATP to form (presumably) formylglycinamidine ribotide in the biosynthesis of purine. This effect has been independently confirmed by Tomisek and co-workers, who have found that azaserine causes the intracellular accumulation of formylglycinamide riboside and ribotide in *E. coli* (56).

Sulfur-Containing Amino Acids

Oxidative catabolism of cysteine.—The frequently-postulated oxidation of cysteine to cysteinesulfinic acid has been directly demonstrated for the first time by Chapeville & Fromageot (57), who isolated the S^{35}-labeled product after injection of the intact rat with S^{35}-cysteine. By recovering S^{35}-cysteinesulfinate from rabbit kidney preparations incubated with $S^{35}O_2$, pyruvate, and glutamic acid (58), the same authors have shown an apparent reversibility of a later step, the cleavage of sulfinylpyruvate to pyruvate and SO_2.

The pathway of cysteine oxidation to taurine has been investigated further. The decarboxylation of cysteinesulfinate to hypotaurine (59, 60) [a reaction dependent on pyridoxal phosphate (61, 62)] and oxidation of the latter to taurine (63) have been shown to occur enzymatically. Nevertheless, there is some evidence that this is not the main pathway of taurine formation in the rat [see discussion by Cavallini *et al.* (60)]. A second possibility has seemed to be the enzymatic decarboxylation of cysteic acid to taurine (61, 62); however, the enzyme which produces cysteic acid by oxidation of cysteinesulfinate is absent from many tissues and is of low activity when present (63a), so that this pathway must certainly be a minor one.

Taurine can be further metabolized to carbamyltaurine and guanidotaurine in the rat [Thoai *et al.* (64)]; the authors suggest a synthesis paralleling that of arginine from ornithine.

Transsulfuration.—Cavallini and co-workers (65) have explored a new possibility which concerns the origin of the cysteinamine (mercaptoethylamine) moiety of coenzyme A. This follows upon the general failure to demonstrate a direct decarboxylation of either cysteine or cystine [the enzyme known as cystine decarboxylase appears to act only on cystine-disulfoxide (see 66)]. They propose that cysteine might condense with ethanolamine to form S-aminoethylcysteine (AEC), followed by cleavage of AEC to cysteinamine and serine. Chemically-synthesized AEC was injected into rats; the products found in the urine included cystamine (oxidized cysteinamine), the α-N-acetyl derivative of AEC, lanthionamine (the decarboxylation product of AEC), and taurine. Since carbon-labeled compounds were not used, however, and control experiments with injected cysteine were not included, it is difficult to see how one can exclude the possibility that the AEC was cleaved back to a form of cysteine, which then was metabolized to cystamine by an unknown decarboxylative route.

The well-known transsulfuration of methionine with serine to yield

cysteine via cystathionine has been studied now in intact mammals using S^{35}-methionine (67) and methionine-2-C^{14} (68). The latter experiment provides the first direct demonstration of the formation of homoserine as a product of cystathionine cleavage.

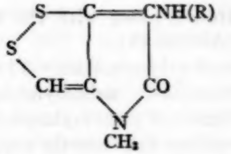
Transmethylation.—The enzyme which transfers a methyl group from betaine to homocysteine to form methionine has been prepared from pigeon liver [Sloane *et al.* (69)] and from mammalian liver [Ericson *et al.* (70, 71)]. Free homocysteine, not S-adenosyl-homocysteine, appears to be the acceptor (71), although in the opposite direction the demethylation of methionine to homocysteine proceeds through S-adenosyl-methionine and S-adenosyl-homocysteine. Erickson, Williams & Elvehjem (71) have prepared a very useful diagram which summarizes the principal routes of transmethylation in animal tissues.

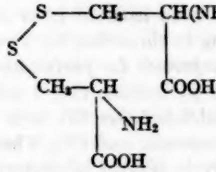
The metabolism of S-methyl-methionine has been further investigated. This compound was shown by Shapiro (72) to transfer one methyl group to homocysteine, thus forming two molecules of methionine, in extracts of *Aerobacter aerogenes.* Schlenk & DePalma (73) came to the same conclusion following upon experiments which showed that S-methyl-methionine is not as good a source of S-adenosyl-methionine and methylthioadenosine as free methionine in intact yeast, but is a much better source than methionine if stoichiometric amounts of homocysteine are added. However, S-methyl-methionine plus homocysteine yielded more adenosine derivative than could be obtained with twice the molar equivalent of methionine, and the authors suggest that S-methyl-methionine may donate a methyl group directly to an adenosine derivative without having to go through free methionine.

Rachele *et al.* (74) have issued a warning concerning the interpretation of experiments with doubly-labeled compounds prepared by mixing deuterium-labeled and C^{14}-labeled materials. They found that methionine methyl groups in the form of $C^{14}D_3$ are metabolized at only 60 to 80 per cent of the rate at which $C^{14}H_3$ groups are metabolized. Thus, if separately labeled compounds (e.g., R-$C^{12}D_3$ and R-$C^{14}H_3$) are mixed together to make a doubly labeled "compound," methylated products may show altered D:C^{14} ratios even when the methyl groups are transferred intact.

Reactions with formaldehyde.—Both homocysteine (75) and cysteine (76) can react with formaldehyde to form cyclic compounds, *m*-thiazane-4-carboxylic acid and thiazolidine-carboxylic acid respectively. The reaction with homocysteine was reported to proceed nonenzymatically, the cyclic product then being metabolized. Thiazolidine-carboxylic acid is metabolized by mitochondria by dehydrogenation and hydrolysis to N-formylcysteine (76).

Miscellaneous.—Two new antibiotics, thiolutin and aureothricin (IV), are reported to have the following structure [Celmer & Solomons (77)]. The reviewer finds their resemblance to cystine (V) noteworthy and suggests a metabolic relationship in their formation:





IV Thiolutin ($R = CH_3 \cdot CO—$) V Cystine

Aureothricin ($R = CH_3 \cdot CH_2 \cdot CO—$)

Aspartic Acid, Homoserine, and Threonine

Interconversion and biosynthesis.—The three enzymes concerned with the conversion of aspartic acid to homoserine have now been described in detail by their discoverers, Black & Wright (78). The enzyme system which converts homoserine to threonine, studied earlier in extracts of *E. coli* by G. N. Cohen and his collaborators [see review by Davis (3)], has now been prepared from yeast by Watanabe & Shimura (79). The latter report that the enzyme is widely distributed in microorganisms but is absent from rat kidney and liver. The yeast enzyme system requires ATP, as was found true for the *E. coli* system also.

Although it is clear from studies with auxotrophic mutants that the above is the main pathway for synthesis of threonine, there is ample evidence that a minor, alternative, route exists. Meinhart & Simmonds (24), for example, found that protein threonine had considerably less specific radioactivity than protein aspartate in *E. coli* grown in the presence of certain C^{14}-labeled substrates; they suggest a reversal of the now well-known cleavage of threonine to glycine as the second pathway. This finding may be contrasted with the results of isotopic competition experiments in *E. coli* (23), which showed that exogenous aspartate suppressed the incorporation of radioactivity into threonine to the same extent as into aspartate itself. The difference in results may well be attributable to the very different experimental conditions used; the latter experiments, which lasted for one generation of cells undergoing exponential growth, may be more significant for normal biosynthesis. It is noteworthy that, although threonine is an essential amino acid for the mammal, extracts of mammalian liver can synthesize threonine from glycine plus acetaldehyde (80); the isomer formed was not identified.

Tomlinson (81) isolated the protein amino acids from *Clostridium kluyveri* grown on $C^{14}O_2$ and found that while the aspartate carboxyls were equally labeled, the gamma carbon of threonine had only 75 per cent as high a specific activity as the carboxl group. He suggested that part or all of the threonine could have been formed by a route involving the known condensation of formaldehyde and pyruvate to make the keto acid corresponding to homoserine. There seems to be no direct evidence for this pathway, however. The keto acid postulated as an intermediate, α-keto-γ-hydroxybutyrate,

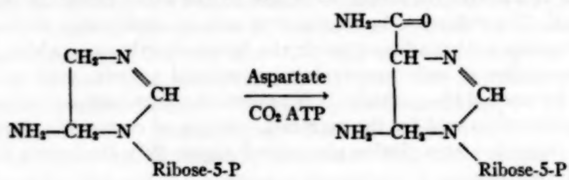
has recently been isolated from plant material, along with the keto acid corresponding to threonine, by Virtanen & Alfthan (82).

Role of aspartate in purine and pyrimidine biosynthesis.—The biosynthesis of the pyrimidine ring from ureidosuccinate (carbamylaspartate) is now well established (see 83), as is the formation of carbamylaspartate from aspartate, ammonia, and CO_2. The latter reaction has recently been studied enzymatically in several laboratories. Smith & Stetten (84) found that rat liver forms C^{14}-orotic acid from citrulline labeled in the ureide carbon and proposed that carbamylaspartate is formed by the splitting of argininosuccinate, the condensation product of citrulline and aspartic acid. However, Lowenstein & Cohen found that the radioactivity incorporated into carbamylaspartate, when a rat liver preparation was incubated with C^{14}-labeled carbamyl donor and aspartate, was not appreciably depressed by addition of unlabeled citrulline (85). From this and other evidence, they conclude that aspartate receives the carbamyl group directly. Since it now appears likely that the ultimate carbamyl donor is carbamylphosphate (see section below on citrulline synthesis), the reaction sequence can be written:

$$NH_3 + CO_2 + ATP \rightarrow \text{carbamyl-P} \xrightarrow{+\text{aspartate}} \text{carbamylaspartate} + P$$

Reichard & Hanshoff (86) have also demonstrated formation of carbamylaspartate from carbamyl donor plus aspartate by enzyme preparations from liver and from *E. coli.* The ability of citrulline to donate the ureide carbon to orotic acid is explained by the finding that citrulline can be decomposed with the formation of carbamylphosphate itself [Reichard, Smith & Hanshoff (87)].

Aspartate also plays a role, as yet poorly understood, in purine biosynthesis. Buchanan, Levenberg and co-workers (44, 46) have shown that a pigeon-liver preparation converts 5-amino-imidazole-ribotide to the carboxamide derivative with the help of aspartic acid, CO_2, and ATP:



The new carboxamide derivative ultimately becomes carbon-6 and nitrogen-1 of purine, which are known from uric acid degradation studies to be derivable from CO_2 and the amino group of aspartic acid respectively (see 83). Wahba, Ravel & Shive (88) suggest that the CO_2 first becomes fixed into a compound which is also derivable from aspartate; this is proposed to account for their observation that a strain of *Lactobacillus arabinosus* can use either aspartate or CO_2 or amino-imidazole-carboxamide, in place of purine, for growth. In the absence of biotin in the medium, CO_2 is no longer active but aspartate remains so.

Formation of glutamic acid.—Two interesting sidelights on the formation of glutamic acid from α-ketoglutarate have appeared. Tomlinson (89) degraded the glutamic acid from *C. kluyveri* grown on C^{14}-labeled substrates and found that the α-ketoglutarate could have arisen via the tricarboxylic acid cycle only if aconitase dehydrated citric acid on the side of the central carbon atom opposite to its customary point of attack. This type of aconitase action had previously been deduced by Corzo & Tatum (90) to take place in the formation of itaconic acid by *Aspergillus terreus*.

Fincham (91) has reported that a mutation which deprives *N. crassa* of glutamic dehydrogenase confers on the mutant a nonspecific requirement for α-amino nitrogen. The reviewer infers that the reductive amination of α-ketoglutarate is the only direct channel for the conversion of ammonia to amino nitrogen in this organism and that transaminations and "side-chain" modifications must account for the formation of all other amino acids.

Formation of ornithine.—Vogel *et al.* (25, 92, 93) have continued to marshal evidence that two different pathways of ornithine formation exist in different organisms. The enterobacteria, including *E. coli*, appear to convert glutamate to ornithine via acetylated intermediates, the final step being the deacetylation of N-acetylornithine by acetylornithinase. N-acetylornithine has also been isolated from green plants (94). *Bacillus* species and higher fungi, however, lack acetylornithinase and instead have an active ornithine-δ-transaminase, which can cause formation of ornithine from free glutamic semialdehyde. This transamination is extremely difficult to demonstrate in the direction of ornithine formation (95), probably because glutamic semialdehyde spontaneously cyclizes to a dehydro form of proline; nevertheless, it is conceivable that in a steady state system there might be sufficient free semialdehyde to permit synthesis of ornithine, by transamination, at an adequate rate. The reader is referred to the original papers and to a lengthy discussion by Davis (3) for many interesting, not to say conflicting, speculations concerning this proposed metabolic path.

Conversion of ornithine to citrulline.—Grisolia, Cohen, *et al.* have worked for some years on the isolation of the active carbamyl donor ("compound X," "intermediate") formed from CO_2, ammonia, ATP, and N-acetylglutamic acid. The latter was believed to be required in stoichiometric amounts and thus to be incorporated into the compound (96). Jones, Spector & Lipmann, however, have now synthesized carbamylphosphate (CAP) and have shown that it can enzymatically transfer the carbamyl group to ornithine directly (97). The question thus was raised whether Compound X is not actually CAP itself. Grisolia, Wallach & Grady (98, 99) conclude that it is not, based on a comparison of the properties of CAP and of Compound X, but Marshall, Hall & Cohen (100) state that acetylglutamate is catalytic, not stoichiometric, in the formation of Compound X from ammonia CO_2, and ATP and find "X" to be identical with CAP in every respect. They report that a bacterial enzyme preparation can synthesize Compound X without participation of any glutamate derivative. These discrepancies appear to have been resolved by Reichard, Smith & Hanshoff (87), who find

that mitochondrial extracts form two different active carbamyl donors from ammonia, CO_2, ATP, and C^{14}-acetylglutamate (or from citrulline, ATP, and acetylglutamate). One of these is CAP and the other, presumably Compound X, is an unknown compound which contains an acetylglutamate residue. The requirement for N-acetylglutamate in CAP synthesis leads them to conclude that Compound X is a precursor of CAP, which then acts as the ultimate carbamyl donor. Compound X is considered to consist of CAP bound in some manner to the glutamate derivative.

Breakdown of citrulline.—Krebs, Eggleston & Knivett (101) have analyzed the breakdown of citrulline to ornithine, CO_2, and ammonia by mammalian liver. This reaction can take place by phosphorolysis or by arsenolysis; if both phosphate and ADP are present, ATP is formed. Ornithine competitively inhibits both arsenolysis and phosphorolysis, and phosphate competitively inhibits arsenolysis. Phosphorylated intermediates were not detected. A mechanism which can account for all these observations was proposed, similar to that suggested by Webster & Varner (102) for synthesis of glutamine, and by Snoke (103) for the synthesis of glutathione. The proposed mechanism involves formation of a phosphate-enzyme-citrulline complex, which first splits off ammonia and CO_2, and then ornithine. The remaining enzyme-phosphate complex can donate an energy-rich phosphate group to ADP or can be hydrolyzed to liberate inorganic phosphate. Arsenate can replace phosphate, but the enzyme-arsenate complex which is formed always hydrolyzes.

An alternative explanation, suggested to the reviewer by Mr. Richard Yates, is that the citrulline-decomposing enzyme carries out a transcarbamylation from citrulline to inorganic phosphate, forming CAP. The latter can then hydrolyze to CO_2, ammonia, and inorganic phosphate, or can donate a phosphate residue to ADP. Transcarbamylation to arsenate would form an unstable carbamylarsenate, breaking down spontaneously to CO_2, ammonia, and arsenate. This hypothesis would seem to fit all of the observations of Krebs *et al.*, as well as with their comment that "the evidence suggests that the reactions are connected with the enzyme system responsible in the intact liver for the synthesis of citrulline from ornithine, CO_2 and ammonia." Their failure to detect a phosphorylated intermediate may have resulted from the fact that the reaction they were studying has an equilibrium favoring formation of citrulline rather than its breakdown.

Conversion of ornithine and arginine to other products.—Experiments with C^{14}-labeled ornithine have confirmed the belief that this compound is a precursor of the pyrrolidine ring of nicotine (104). Arginine has been implicated as the source of the guanidine groups of streptomycin (105).

Both arginine and ornithine (as well as lysine) are substrates for a dehydrogenase of turkey liver which is relatively specific for the basic amino acids. L-Arginine is oxidized to α-keto-δ-guanidovaleric acid; L-ornithine becomes α-keto-δ-aminovaleric acid, which cyclizes to Δ'-pyrroline-2-carboxylic acid [Boulanger & Osteux(106)]. The latter compound can also

be formed from glutamic semialdehyde and is the principal product of the action of ornithine-δ-transaminase (see p. 357).

Isoleucine, Valine, and Leucine

Biosynthesis of isoleucine and valine.—A number of papers have appeared which in general confirm and extend the material on this subject which has been summarized in recent reviews (2, 3, 6, 8). Strassman, Thomas & Weinhouse (107) have published the complete report of their experiments which demonstrate the origin of valine from two molecules of pyruvate by a pathway which involves an intramolecular rearrangement. This conclusion, based on experiments with yeast, has been extended to the bacterium *Aerobacter aerogenes* by Rafelson (108). Adelberg, Coughlin & Barratt (109) have published the details of their experiments which invalidate the earlier suggestion by Tatum & Adelberg (110) that isoleucine and valine are formed from a common intermediate in *Neurospora;* and Adelberg (111) has presented the full account of the tracer experiments which demonstrate the origin of isoleucine from threonine by a pathway again involving an intramolecular rearrangement. The origin of valine from pyruvate, rather than from an isoleucine precursor, has also been established in *E. coli* by Cohen & Hirsch (112), who found that suspensions of cells convert added pyruvate to the keto acids corresponding to valine and leucine, both with the wild-type strain and with mutants blocked in the biosynthesis of isoleucine precursors.

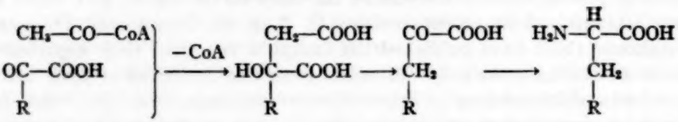
Umbarger (113) has correlated the ability of mutants of *E. coli*, but not of *Neurospora*, to substitute D-threonine for L-isoleucine as a growth factor, with the much higher activity in *E. coli* of D-threonine deaminase. The deaminase forms α-ketobutyric acid, which is readily used as a precursor of isoleucine. Umbarger & Brown (114) have studied the peculiar valine sensitivity of strain K-12 of *E. coli* and have concluded, on the basis of experiments which show that isoleucine reverses valine inhibition noncompetitively, that valine blocks isoleucine synthesis in this strain. Inhibition experiments with an isoleucineless-valineless mutant of this strain led them to conclude further that isoleucine antagonizes valine uptake by *E. coli* cells. However, their growth data are entirely in the form of 24-hr. optical density readings, and the significance of such data is open to question (see pages 365 to 67).

Biosynthesis of leucine.—Strassman *et al.* (115) grew *Torula utilis* in the presence of variously-labeled C^{14}-acetates and lactates, then isolated and degraded the leucine and valine in each case. They found the distribution of radioactivity in positions 3, 4, 5, and 5′ of leucine to be almost identical with that in positions 2, 3, 4, and 4′ of valine while the carboxyl and alpha carbons of leucine were clearly derived from acetate as a unit. These data beautifully confirm the results of Abelson (23), who deduced from isotopic competition experiments that leucine is synthesized from the corresponding keto acid, which in turn arises from the keto acid precursor of valine by decarboxylation and addition of an acetate unit. Strassman *et al.* propose that a series of reactions analogous to part of the citric acid cycle take

place; they had already proposed a similar series for the origin of α-aminoadipic acid from α-ketoglutarate in the biosynthesis of lysine by yeast (116), so that their views can be generalized as indicated in Table I.

TABLE I

BIOSYNTHESIS OF LEUCINE



Acetyl acceptor	R	Final product
α-Keto-isovalerate	$—CH(CH_3)_2$	Leucine
α-Ketoglutarate	$—CH_2\cdot CH_2\cdot COOH$	α-Aminoadipic acid
Oxalacetate	$—CH_2\cdot COOH$	Glutamic acid

The proposed intermediates in lysine and leucine biosynthesis remain to be demonstrated. Reiss & Bloch (117), on the basis of very similar experiments, agree that the isobutyryl portion of leucine may arise, like valine, by rearrangement of the condensation product of two molecules of pyruvate, and that the first two carbons come directly from acetate. However, the specific radioactivities which they found for carbons 3 and 4 differed sufficiently from those found by McManus (118) for valine carbons 2 and 3 in the same cells, to convince them that the keto acid precursors of valine and leucine arise by different pathways. The reviewer notes, however, that the specific activity of valine carbon 3 was calculated by difference rather than determined directly (118); as the same is true of the data for leucine carbon 3 in the experiments of Reiss & Bloch (who state that "the value assigned to C-3 may be considerably in error"), the rejection of the Abelson-Strassman hypothesis does not appear warranted on the basis of these data alone.

Catabolism of branched chain amino acids.—This subject has been recently reviewed by Coon (119) in a symposium on cholesterol metabolism. The catabolism of the branched chain amino acids leads to the formation of various branched chain fatty acids, some of which (e.g., β-hydroxy-β-methylglutaric acid) have been implicated as precursors of the isoprene building blocks for steroid synthesis. However, as Coon points out, the branched precursors of steroids do not come from pre-formed amino acids, but rather from straight-chain acids (e.g., acetoacetate) by reactions which are the reverse of the final steps in the breakdown of the branched amino acids.

Conversion of valine to other cell products.—Arnstein & Clubb (120), by use of C^{14}, N^{15}-labeled valine, have shown that the intact carbon skeleton of valine is used for penicillin synthesis but that the amino group becomes lost. The keto acid precursor of valine, α-keto-isovalerate, has been implicated in pantothenic acid synthesis (121). Nelson *et al.* (122) have now carried out

the enzymatic synthesis of what appears to be the α-keto acid analogue of pantoic acid, by incubating extracts of *E. coli* with α-keto-isovalerate, formaldehyde, divalent metal ions, and tetrahydrofolic acid:

$$H_2CO + CH(CH_3)_2 \cdot CO \cdot COOH \xrightarrow{\text{THFA}} CH_2OH \cdot C(CH_3)_2 \cdot CO \cdot COOH$$

Histidine

Biosynthesis.—Ames & Mitchell (123) have now published a complete account of their work which establishes the sequence: imidazoleglycerol phosphate (IGP)→imidazoleacetol phosphate→histidinol phosphate→histidinol→histidine, in *Neurospora.* The origin of IGP is still something of a mystery; the clues so far include the derivation of the terminal carbon in the side chain from acetate methyl and the derivation of carbon 2 of the ring from formate (see 3). The suggestion that the five-carbon chain of IGP comes from pentose phosphate still seems the most plausible in spite of the recent observations of Luzzati *et al.* (124, 125) on an *E. coli* mutant which is able to grow on either purine or histidine. Their work indicates that purine can serve as a minor source of histidine for *E. coli,* but the data do not support purine as a normal biosynthetic precusor of IGP. Conversely, their tracer experiments with the same mutant indicate that histidine can furnish a portion of the purine molecule, although the normal biosynthesis of the purine ring in *E. coli* quite clearly proceeds via formylglycinamide ribotide (see p. 351).

Adams (126, 127) has synthesized histidinal, the aldehydic compound which had been unsuccessfully sought as an intermediate in the DPN-linked oxidation of histidinol to histidine. His enzyme studies strongly implicate histidinal as the true intermediate and suggest that both oxidations are catalyzed by the same enzyme.

Catabolism.—The catabolic pathway, histidine→urocanic acid→α-formamidinoglutaric acid, has been established in mammalian liver [see Miller & Waelsch (128)]. The indicated product is further degraded to glutamate plus formamide in *Clostridium tetanomorphum* (129) and in *A. aerogenes* (130), and to N-formylglutamate plus ammonia in *Pseudomonas fluorescens* (131). N-formylglutamate is hydrolyzed to glutamate plus formate by the latter organism (131).

A second pathway of histidine degradation has been studied by Hayaishi and co-workers (132, 133, 134). This path involves the steps: histidine→histamine→imidazoleacetaldehyde→imidazoleacetic acid→formylaspartic acid→aspartic acid + formate. The oxidation of imidazoleacetate is DPN-linked (132). A *Pseudomonas* species was found to produce a formylase specific for formylaspartate when grown on imidazoleacetate and a different, nonspecific, formylase (attacking formylglutamate and acetylaspartate as well), when grown on histidine (133). Both histamine and imidazoleacetic acid are converted to imidazoleacetic acid riboside by the rat *in vivo* (134, 135); the reviewer suggests that the close structural relationship between

this histidine catabolic product and certain purine precursors (46) may explain the limited ability of histidine to furnish part of the purine skeleton in *E. coli*, as discussed above.

Histidine as coenzyme.—Larner & Gillespie have studied the coenzyme action of histidine in reactions catalyzed by certain intestinal carbohydrases and propose a mechanism of action (136). They list six other systems in which histidine has been reported to be catalytic.

Lysine, Hydroxylysine, and Diaminopimelic Acid

Biosynthesis of lysine and hydroxylysine.—There is strong evidence that the principal route of lysine synthesis in *E. coli* is through decarboxylation of diaminopimelic acid (DAP) which in turn arises, by reactions as yet unknown, from aspartic acid (see review, 3). Dewey, Work and their collaborators have now published a series of papers on DAP decarboxylase, which is shown to be a constitutive pyridoxal-phosphate-containing enzyme present in many bacteria (137, 138, 139). However, the activity of acetone-dried cells varied greatly between different strains of coliform bacteria, and no enzyme was detected in one strain of *Proteus vulgaris*, in one of *Bacillus subtilis*, or in *Streptococcus faecalis* (138). The authors seem to feel that this argues against a general role for DAP in lysine biosynthesis by bacteria, although under their experimental conditions (cells grown in peptone medium and harvested at an arbitrary time) there is no assurance that the harvested cells were synthesizing lysine at appreciable rates. Indeed, Roberts *et al.* (140) have found that the presence of lysine in the medium severely depresses the rate of endogenous lysine synthesis (but not DAP synthesis) in *E. coli*, which might well reflect a direct effect on DAP decarboxylase activity. As far as the reviewer can determine, all of the DAP decarboxylase studies have been done with cells grown in lysine-containing media and, therefore, cannot be considered meaningful for analysis of lysine biosynthesis.

In yeast, lysine synthesis is believed to take place from α-ketoadipic acid via α-aminoadipic acid; α-ketoadipate is formed from the succinyl moiety of α-ketoglutarate plus acetate (see 3, also page 360 of this review). Abelson & Vogel (25) have now extended this conclusion to *N. crassa*, with the one difference that the C_2 fragment appears not to be derivable from acetate in this organism.

Sinex & Van Slyke (141) have fed uniformly labeled C^{14}-L-lysine to growing rats; the lysine and hydroxylysine which they isolated from the skin collagen had identical specific radioactivities and identical ratios of activity between the carboxyl groups and remainder of the molecules. Thus the biosynthesis of hydroxylysine by oxidation of lysine can be inferred.

Catabolism of lysine.—Two routes of lysine catabolism have been further studied. Rothstein & Miller earlier provided evidence for the following pathway in the rat: lysine→α-aminoadipic acid→glutaric acid→α-ketoglutaric acid; a secondary pathway, operating only when excess lysine was administered, appeared to be formation of the α-keto acid followed by oxida-

tive decarboxylation to δ-aminovalerate, which would itself be oxidized to glutaric acid (142). These authors now find that δ-aminovalerate-δ-C^{14} appears to be catabolized over the same route indicated for lysine-6-C^{14}, again suggesting the existence of the second path for lysine breakdown (143). δ-Aminovalerate is capable of transamination in lysine-adapted bacteria (143a), yielding glutaric semi-aldehyde. Such a reaction might well be involved in the catabolic pathway discussed above.

In *Neurospora* Schweet, Holden & Lowy (144) have shown that lysine catabolism starts with its oxidative deamination to α-keto-ε-aminocaproate, which spontaneously cyclizes to dehydropipecolic acid. This intermediate is next reduced to pipecolic acid. Another product of the *Neurospora* attack on lysine is an N-substituted derivative of α-hydroxy-ε-aminocaproic acid, which was tentatively proposed to originate from pipecolic acid.

Metabolism of diaminopimelic acid.—The decarboxylation of DAP has already been discussed in the section on lysine biosynthesis. DAP has been found by Work to be susceptible to the L-amino acid oxidases of snake venoms and of *Neurospora* (145); the different isomers (for preparation, see 146) and amides of DAP are attacked at different rates.

Perry & Foster (147) prepared DAP totally labeled with C^{14}, and added it to spore-forming cultures of *Bacillus* species. These cells form spores which contain 5 per cent of their weight as dipicolinic acid (pyridine-2,6-dicarboxylic acid); this could theoretically be formed from DAP by deamination, ring closure, and dehydrogenation. The dipicolinate which they isolated from the spores had a specific radioactivity about 3 per cent that of the DAP administered and four times the highest specific activity among 12 amino acids also isolated. The results are not incompatible with the view that DAP is a normal precursor of dipicolinic acid, but no inference can be drawn about the mechanism. Among the amino acids, leucine and valine had the highest specific activities, with alanine and aspartate next, but much lower. Since leucine and valine are uniquely derived from a branched-chain condensation product of two molecules of pyruvate (see page 359 et seq.), it would be of considerable interest to know the route by which the carbon of DAP enters these amino acids.

Aromatic Amino Acids

The biosynthesis of dehydroshikimic acid, a precursor of the aromatic ring, was earlier demonstrated to take place from sedoheptulosediphosphate (SDP) via dehydroquinic acid (see 3). Isotopic data, however, indicate that SDP does not retain its carbon chain intact. The full explanation has now been provided by Srinivasan, Katagiri & Sprinson (148); they found that cell-free preparations of an *E. coli* mutant convert D-erythrose-4-phosphate plus phosphoenolpyruvate to dehydroshikimic acid in good yield. Inhibition studies indicate that when SDP is the substrate, it is split to tetrose phosphate plus dihydroxyacetonephosphate and that the latter is converted to phosphoenolpyruvate via 3-PGA. Since tetrosephosphate and phosphoenol-

pyruvate are both generated during the oxidation of glucose (149), SDP does not appear to be on the main pathway.

In the biosynthesis of tryptophan, the mechanism of the conversion of anthranilic acid to indole has long remained a mystery, except for the knowledge that the carboxyl group is lost. This problem has now been opened to direct experimental attack by Yanofsky, who has found that extracts of *E. coli* will carry out the conversion in the presence of phosphoribosylpyrophosphate. Ribose labeled in the 1 position with C^{14} yields indole with label in the carbon atom adjacent to the pyrrole nitrogen; the question whether phosphoribosylpyrophosphate donates a C_2 fragment, or whether a ribotide of anthranilate is first formed, remains to be answered (150, 151).

Tryptophan catabolism.—Several papers have been published which implicate various vitamins as catalysts in the breakdown of tryptophan. Shanmuga Sundaram *et al.* (152) report that a biotin antimetabolite inhibits the growth of a nicotinicless *Neurospora* mutant on tryptophan but not on N-formylkynurenine or later nicotinic acid precursors, and that this inhibition is reversed by biotin. They conclude that biotin is required for the oxidation of tryptophan to formylkynurenine. It is strange that no other biotin-requiring reactions become inhibited under their experimental conditions; it would be interesting to know the effect of the biotin antimetabolite on the wild-type strain. Dalgliesh (153) could find no evidence of a biotin role in the mammalian breakdown of tryptophan, in that biotin-deficient animals showed normal patterns of metabolite excretion. The Dalgliesh group (154) also questioned the conclusion of Henderson *et al.* (155) that riboflavin is involved in the oxidation of kynurenine to 3-hydroxykynurenine, because they found that riboflavin-deficient animals showed an increased xanthurenic acid excretion when given tryptophan. Henderson's group appears to have explained this anomaly by showing that administered kynurenine is converted to xanthurenate in much greater yield than is administered tryptophan, and that the riboflavin-deficient animals probably accumulate kynurenine. Thus the amount of xanthurenate excreted is not necessarily a true measure of the rate of oxidation of kynurenine to the hydroxy derivative (156). Finally, Dalgleish has found that thiamine deficiency blocks the excretion of kynurenine metabolites by pyridoxine-deficient rats and that the effect is reversed by thiamine. Thiamine appears to act either directly in the oxidation of tryptophan to N-formylkynurenine or in the formation of the peroxidase-oxidase system (157).

Another catabolic pathway for tryptophan has been explored by Weissbach, Udenfriend and co-workers. *Chromobacterium violaceum*, which forms a pigment consisting of a derivative of 5-hydroxyindole, oxidizes tryptophan to 5-hydroxytryptophan (158). The latter compound is decarboxylated to 5-hydroxytryptamine (serotonin) by an enzyme obtained from hog kidney or guinea pig kidney (159). Serotonin is found in practically all animal species, which presumably, therefore, can also carry out the formation of 5-hydroxytryptophan from tryptophan (160). Another pathway for 5-hydroxytryptophan metabolism may be its further oxidation to 5-hydroxyky-

nurenine; the latter compound has been synthesized by Makino & Takahashi (161) who find that it is deaminated to 6-hydroxykynurenic acid by liver homogenates from rabbits, toads, or mice; 4,6-dihydroxyquinoline is also formed.

Catabolism of other amino acids.—Dische & Rittenberg (162) found that rat liver slices form carboxy-labeled fumaric and malic acids from phenylalanine-4-C^{14}. This neatly substantiates the scheme for phenylalanine catabolism proposed by Ravdin & Crandall (163) on the basis of experiments with the intermediate homogentisic acid. Phenylalanine labeled in position 4 becomes homogentisic acid labeled in position 5 (side-chain migration), and the latter is cleaved first to maleylacetoacetate and later (after isomerization) to fumaric and acetoacetic acids (see 164).

Dalgliesh (165) has applied the system of Udenfriend *et al.* (166) to the nonenzymatic hydroxylation of aromatic amino acids and finds that many hydroxy derivatives are formed. Since the required substances (ferrous ions, ascorbate, a chelating agent, and oxygen) are often available in physiological systems, it was proposed that such reactions may become important when the faster enzymatic ring oxidations become blocked.

New and Unusual Amino Acids

Thanks to Virtanen and his associates, the following amino acids have been demonstrated in extracts of green plants: γ-hydroxyglutamic acid (167), a compound tentatively identified as γ-methyl-γ-hydroxyglutamic acid (168), and a cyclic compound made up of three molecules of homoserine (169). This group has also shown that cells of *E. coli* decarboxylate γ-hydroxyglutamic acid to the novel amino acid, α-hydroxy-γ-amino-*n*-butyric acid (170).

Free allohydroxy-L-proline has been reported to occur in leaves of sandal (171), and S-methylcysteine sulfoxide in cabbage (172).

Measurement of Bacterial Growth as an Index of Amino Acid Metabolism

So many papers appear each year in which amino acid metabolism is analyzed through experiments on bacterial growth that a comment on this method seems highly pertinent. This type of analysis has become particularly popular in studies on amino acid analogues and on the utilization of amino acids, their precursors, and their peptides, as growth factors.

In the *Annual Review of Microbiology* for 1949, Monod presented an essay on "The Growth of Bacterial Cultures" (173), which should be carefully studied by anyone intending to apply quantitative bacteriology to biochemical problems. Monod clearly defines the meaningful growth parameters, which are as follows: "total growth" (this would include such variants as concentration of factor required for maximal growth, or for half-maximal growth, or the ratio of cell yield to nutrient supplied); "exponential growth rate" (generation time during the logarithmic growth phase); and "lag time," which is conveniently measured as the difference in the number

of cell divisions between observed and "ideal growth" as determined at some point of time during the exponential phase.

Each of these three parameters has a different physiological significance, none of which is likely to be simple. "Total growth" may be determined by such factors as exhaustion of the limiting nutrient (which in turn is directly proportional to the efficiency of its utilization for synthetic processes), accumulation of toxic products, or unfavorable changes in pH. "Growth rate" is determined in complex fashion by a large number of different rate-determining steps in the steady state metabolism, or possibly by the rate of penetration of factor into the cell. "Lag time" may have an even more complex basis, depending as it does on the development of the steady state through changes in catalytic power, in concentration of metabolites, or both.

If the biochemist properly measures the effects of his compounds on one or another of these parameters, his job of interpretation will be difficult enough. But if he measures all of his effects in terms of "optical densities at 24 hours at 37°C" or some such arbitrary figure, he cannot possibly know which of the growth parameters he has measured. For example, the following graph (Fig. 1) portrays some typical growth curves, where A, B, and C

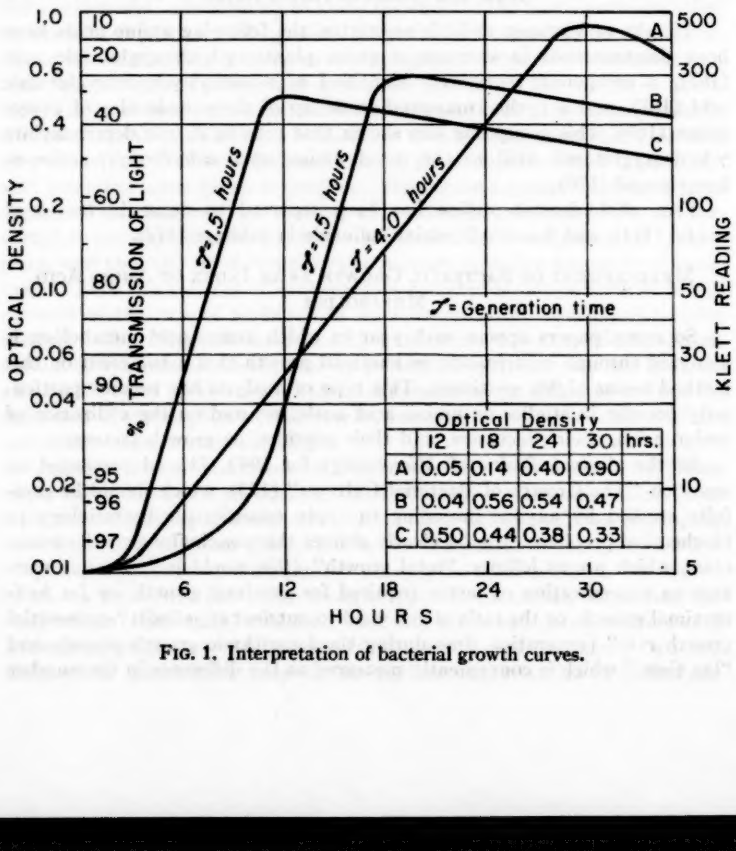


FIG. 1. Interpretation of bacterial growth curves.

represent different experimental conditions (e.g., different growth factors, or factors at different concentrations, or inhibitors supplied in different forms): Consider the different interpretations which would be put forth if the effects of conditions A, B, and C were compared at 12, 18, or 30 hours instead of at 24!

Obviously, no determination of growth made at one arbitrary time can have much meaning. For those who feel more at home with manometric techniques than with bacterial physiology, it need only be pointed out that such methods of reporting data are no more useful than Warburg experiments would be, if the latter were reported solely in terms of "manometer readings at 45 minutes." Only by plotting the entire growth curves as in Figure 1 could it be discovered that condition A provides the highest total growth, condition C a higher growth rate but less total growth, while condition B induces an extended lag phase lasting the equivalent of several extra generations.

Nevertheless, the use of arbitrary incubation periods is the basis for most of the antagonism and nutrition studies in the current literature (e.g., 88, 114, 174 to 180). For examples of significant bacterial growth measurements on the other hand, the reader is referred to the papers by Luzzati & Guthrie (124) and by Meinhart & Simmonds (47). To quote Monod,

> the time-honored method of looking at a tube, shaking it and looking again before writing down a + or a 0 in the lab-book has led to many a great discovery. Its gradual replacement by determinations of "turbidity at 16 hours" testifies to technical progress, primarily in the manufacture and advertising of photoelectric instruments. This technique, however, is not, properly speaking, quantitative, since the quantity measured is not defined. It might be a rate, or a yield, or a combination of both.

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II. PROTEIN METABOLISM[2]

By M. Rabinovitz

Several reviews on various aspects of protein metabolism have appeared in the treatise, *The Proteins*; Steward & Thompson (181a) cover "Proteins and Protein Metabolism in Plants;" Hughes (171b) reviews "Interstitial Proteins, the Proteins of the Blood Plasma and Lymph;" and Tarver (181c) discusses "Peptide and Protein Synthesis, Protein Turnover." In addition, several comprehensive presentations of work on protein metabolism carried out in various laboratories have been published following the *Symposium on Amino Acid Metabolism* held at the McCollum Pratt Institute in June 1954, and reference to these articles will be made in this review.

Because of the limitation of space, the sections on nutrition and control of protein metabolism were severely constricted, and problems associated with the sparing of amino acid requirements and the metabolism of heterologous proteins were omitted.

Digestion and Nutrition

Utilization of proteins.—The biological value of proteins seems to be in part related to the release of essential amino acids which occurs during peptic digestion [Sheffner, Eckfeldt & Spector (182)]. However, analysis of intestinal contents may be misleading, for sufficient amino acids are released by intestinal autodigestion to prevent any correlation with dietary protein [Nasset, Schwartz & Weiss (183)]. Proteins that are heat-denatured have been found by Viswanatha & Leiner (184) to be better utilized than native protein for growth of *Tetrahymena pyriformis*, a protozoan which has dietary protein requirements similar to those of the rat [Pilcher & Williams (185)]. Fisher & Scott (186) report that those amino acids which are required by the chick in the smallest quantity are used most effectively. This efficiency of utilization may not occur if the limiting amino acid is present in too small proportions, for a deficiency in the diet is reflected to an exaggerated degree in the blood [Almquist (187)]. The growth of chicks fed amino acid rations containing ample levels of essential and nonessential amino acids is stimulated by inclusion of gelatin or its hydrolysate to the extent of 5 per cent of the diet. The biological evaluation of proteins has been reviewed by Allison (188).

Mitchell (189) has presented data supporting Folin's concept of a dichotomy in protein metabolism into endogenous and exogenous types.

Amino acid requirements of mammals.—Shultze (190) found normal reproduction in rats that were fed amino acid rations in place of protein; however, the following generation grew at a subnormal rate. Young rats fed

[2] The following abbreviations are used in this section: ADP for adenosinediphosphate; ATP for adenosinetriphosphate; DNA for deoxyribonucleic acid; RNA for ribonucleic acid.

with lysine-deficient rations remain in positive nitrogen balance [Bothwell, Prigmore & Williams (191)]. Since the retained nitrogen was not found in the blood, the results indicate either a lack of protein breakdown or a cellular retention of amino acids induced by the potential for growth. These authors (192) have also demonstrated that rations deficient in histidine, methionine, and lysine can in part replenish xanthine oxidase, succinoxidase, and choline oxidase in the liver of the protein-depleted rat. Mice that are fed phenylalanine-deficient diets convert less phenylalanine-C^{14} to tyrosine or carbon dioxide [Grau & Steele (193)]. Armstrong (194) reports that the conversion of phenylalanine to tyrosine in the young rat is insufficient for growth.

Rose and collaborators have continued their determination of the amino acid requirements for nitrogen balance in young men. These include in gm./day of the L-amino acid: tryptophan 0.25 (195), phenylalanine 1.1 (196), lysine 0.8 (197), threonine 0.5 and methionine 1.1 (198), leucine 1.1 and isoleucine 0.7 (199). Rose, Coon & Lambert (200) have also reported that a higher caloric intake is required to maintain humans in nitrogen balance with casein hydrolysates or amino acid mixtures than with casein. The specific dynamic action of proteins has been shown by Abelin & Goldstein (201) to be attributable to increased epinephrine secretion.

Steinbock & Tarver (202) reported that high casein diets cause an increased rate of plasma protein formation and degradation in the rat. This is reflected by an increased incorporation of labeled amino acids into the protein of liver slices [Rutman *et al.* (203)].

Amino acid requirements in tissue culture.—Eagle has shown that tissue cultures of both mouse fibroblasts (204) and human carcinoma cells (205) require amino acids which are nonessential in the whole animal. The requirement for cystine has been stressed by Morton & Morgan (206). The possible utilization of intact peptides by tissue culture preparations is discussed in a following section.

Requirements for the formation of specific proteins.—According to Nasset & Gatewood (207) levels of histidine adequate for nitrogen balance in adult male rats are inadequate for maintenance of normal hemoglobin concentrations. Nizet & Lambert (208) have reported a factor in plasma which stimulates hemoglobin synthesis in reticulocytes. This resembles the factor, consisting of 1-deoxy-1-(N-amino acid)-2-ketohexoses, found by Borsook, Abrams & Lowy (209) to stimulate iron absorption and thus the incorporation of radioactive amino acids into reticulocyte hemoglobin. Of interest is the previously mentioned finding of Prigmore *et al.* (192) that rations deficient in certain amino acids can partially replenish various liver enzymes in the protein depleted rat. This may be attributable to a hierarchy of affinities among the many protein-forming systems for limiting amino acid supplies in the deficient animal.

Proteins in embryonic nutrition.—Kemp (210) has demonstrated incorporation of glycine into the peripheral cytoplasm of frog oocytes that are actively synthesizing yolk. In the developing chick egg, Rupe & Farmer

(211) found that the yolk is the sole source of amino acids during the first 250 hr. of incubation. After that time the white supplies the major portion. Kavanau (212) has demonstrated three major and three minor periods of new protein synthesis in the developing sea urchin, *Paracentrotus lividus*. These are coupled to four periods of intense yolk-protein breakdown. Whipple and co-workers (213), by use of lysine-labeled plasma proteins, have found that only 200 to 300 mg. per day of maternal plasma protein reaches the fetus in the pregnant bitch, thus indicating the importance of amino acid transfer during fetal development. Literature on the transport of proteins across fetal membranes has been summarized by Brambell (214).

Absorption and Activation of Amino Acids

Characteristics of amino acid absorption.—Fridhandler & Quastel (215) report that amino acids are actively absorbed by the intestine when present in low concentrations, but that at $0.02M$ or above they are absorbed by diffusion. In agreement with earlier workers [Wilson & Wiseman (216); Agar, Hird & Sidhu (217, 218); Wiseman (219)] they found that L-amino acids but not the D- are actively absorbed and that this active absorption is inhibited by anaerobiosis and dinitrophenol. Shishova (220) has indicated that amino acid absorption by the intestine is stimulated by ATP. Ehrlich ascites cells absorb diamino acids actively, and those with three carbon atoms between the nitrogens are absorbed better than those with four [Riggs, Coyne & Christensen (221)]. Since pyridoxal stimulates uptake, it was suggested that the three-carbon structure may produce the more stable pyridoxylidene derivatives, postulated by Christensen, Riggs & Coyne (222) to occur during active absorption of amino acids. The carcinoma cells from pyridoxine-deficient mice absorb less glycine than those from nondeficient animals; this is partially relieved by pyridoxal. Inhibition of amino acid uptake by deoxypyridoxine (222), however, may be nonspecific, for Fridhandler & Quastel (215) report a similar inhibition of the absorption of glucose and fructose by the intestine. Heinz (223, 224) found that prior saturation of Ehrlich ascites cells with unlabeled glycine increased the subsequent influx of the radioactive amino acid, suggesting that glycine is not in a bound form within the cell. Halvorson, Fry & Schwemmin (225) demonstrated that uptake of amino acids by starved yeast requires energy. In *Neurospora crassa* histidine may not be absorbed effectively in the presence of arginine and lysine [Matheson & Catcheside (226)]. Ionic balance in amino acid absorption by yeast and bacteria has been studied by Davies *et al.* (227). The mode of transport of amino acids into mammalian cells has been reviewed by Christensen (228).

Amino acid activation in peptide synthesis.—Enzymes have been isolated from wheat germ [Webster & Varner (229)] and hog liver [Mandeles & Bloch (230)] which catalyze the formation of γ-glutamylcysteine from glutamic acid and cysteine in the presence of Mg^{++} and K^{+} and with the stoichiometric cleavage of ATP to ADP and inorganic phosphate. The mechanism

of amino acid activation in the formation of glutathione from γ-glutamylcysteine and glycine has been studied by Snoke & Bloch (231) with a yeast enzyme [Snoke (232)] and by Webster & Varner (233) with an enzyme from wheat germ. The latter workers demonstrated an exchange reaction between inorganic P^{32} and ATP which was catalyzed by the enzyme only in the presence of glutamylcysteine; this suggests the following reactions:

$$\text{ATP} + \text{Enzyme} \rightleftharpoons \text{Enzyme-P} + \text{ADP}$$

$$\text{Enzyme} - \text{P} + \text{Glutamylcysteine} \rightleftharpoons \text{Enzyme-glutamylcysteine} + \text{Inorganic Phosphate}$$

The yeast enzyme of Snoke & Bloch, however, did not carry out this exchange with inorganic phosphate and would not bind inorganic phosphate when treated with ATP, although it could transfer the terminal phosphate of ATP to ADP. The mechanisms of action of the enzymes from the two sources may be different. In addition, it now appears generally accepted that amino acid activation for the synthesis of small peptides may bear little relationship to that involved in protein synthesis.

Transpeptidation and amino acid activation.—Attempts to verify the postulated role (234) of γ-glutamyl transpeptidation in energy transfer associated with protein synthesis have proven fruitless. With several tissue preparations, Hendler & Greenberg (235) found poor incorporation of glycine-C^{14} into protein when it was present as γ-glutamylglycine. Incorporation occurred only after hydrolysis of the peptide. Similar negative results have been reported by Krahl (236) with S^{35}-labeled glutathione. Waelsch (236) observed that γ-glutamyl peptides other than glutathione did not support the growth of *Lactobacillus arabinosus* and that labeled glycine was incorporated into liver protein at a faster rate than into liver glutathione (237). Hird & Springell (238) found the hydrolytic and transfer activities of the γ-glutamyl transferase from sheep kidney to run parallel, indicating that amino acids compete with water in an otherwise peptidolytic system. The amide group of glutamine also does not appear to be uniquely involved in catalyzing amino acid transfers, for Barry (239) has shown that glutamine-1-C^{14}-amide-N^{15} is incorporated into casein with retention of both labels. Williams & Thorne (240, 241) have obtained an enzyme from *Bacillus subtilis* which catalyzes the formation of γ-glutamyl peptides from glutamine. *B. subtilis* can both synthesize and hydrolyze a γ-linked DL-polyglutamic acid [Thorne *et al.* (242)], and the transferase studied is associated with enzymes capable of hydrolyzing this polypeptide (240). Its action could therefore resemble the aforementioned γ-glutamyl transferase of sheep kidney and may not represent the polypeptide synthesizing system of the intact cell. In this regard, it would be of interest to apply the observation of Hahn, Wisseman & Hopps (243) that the L(+)-erythro isomer of chloramphenicol inhibits synthesis of the D-glutamic acid polypeptide of *B. subtilis* much as the antibiotic D(−)-threo isomer inhibits protein synthesis from L-amino acids.

Energy relationships in protein synthesis.—Zamecnik & Keller (244)

demonstrated that under anaerobic conditions suitable energy sources can support the incorporation of radioactive amino acids into the microsome-rich fraction of rat liver homogenate. Guanosine triphosphate may be involved in this energy transfer [Keller & Zamecnik (245)]. Rabinovitz, Olson & Greenberg (246) have shown that incorporation of radioactive amino acids into protein of the Ehrlich ascites carcinoma can be supported by anaerobic glycolysis. Incorporation stops abruptly upon glucose depletion. These authors also indicated (247) that amino acids do not compete for activation at the same site, since high concentrations of one amino acid do not interfere with the incorporation of another present in low concentration. A similar failure of one amino acid to compete with another was observed when the energy supply was made limiting with dinitrophenol (246). This specificity of amino acid activation by the Ehrlich ascites cells indicates that such activation is unrelated to the general concentrative uptake of amino acids by these cells [Christensen (228)].

A family of enzymes, members of which couple the activation of individual amino acids with pyrophosphate cleavage from ATP, has been found in rat liver extracts by Hoagland (248, 249). The activated amino acids could exist as enzyme bound adenyl-amino acids (249). These enzymes may be the constituents present in the supernatant fluid of liver homogenate which are essential to the amino acid incorporating system of microsomes [Keller, Zamecnik & Loftfield (250)]. Similar activating systems have been found by DeMoss & Novelli (251) in extracts of several microorganisms including *E. coli*. This species, which has no free amino acid pool, can concentrate amino acids by binding them at specific sites [Cohen & Rickerberg (252); Britten, Roberts & French (253)]. The concentration requires energy and is in the pathway of protein synthesis. Britten *et al.* (253) present evidence that the binding may be by hydrogen bond formation. It may therefore differ from the aforementioned activating system of DeMoss & Novelli.

Peptides as Intermediates in Protein Synthesis

The utilization of peptides of small or intermediate length in protein synthesis has continued to provide an attractive explanation for a variety of biological phenomena. The stimulation of microbial growth by many characterized peptides or unidentified peptide fragments was reviewed by Ehrensvärd (254), and the literature has also been covered by Meinhart & Simmonds (255). Further demonstrations have been made of microbiological systems in which peptides are utilized more efficiently for growth than their constituent amino acids. These include cases where the amino acid is rapidly degraded by the organism but can be utilized for growth when slowly liberated from peptide form. Such is the explanation of Miller, Neidle & Waelsch (256) for the observed preferential utilization of asparagine peptides by *Leuconostoc mesenteroides*. Also, Magasanik (257) reported that histidine induces the formation of degradative enzymes in *Aerobacter aerogenes* and is, therefore, poorly utilized, while acyl derivatives cause no

such induction but slowly liberate histidine in quantities sufficient for growth. In addition, when amino acid antagonists are present in the medium, it appears to be a general rule that better growth occurs if the amino acid is made available in peptide form [Kihara & Snell (258)]. This is a result of competition by the antagonist with its corresponding amino acid for entrance into the cell and the absence of such competition with peptides of this amino acid. It is generally recognized that such evidence has little bearing on the problem of the direct utilization of peptides for protein synthesis.

In attempts to devise critical experiments for demonstrating the utilization of intact peptides, Peters & Snell (259) have shown that the growth of *Lactobacillus delbrueckii* can be inhibited by high concentrations of glycyltryptophan and glutathione. These compounds appears to function as peptide antagonists, for inhibition can be prevented by partial but not complete protein hydrolysates. In another vein, Meinhart & Simmonds (255) have demonstrated that serylglycine is significantly superior to the constituent amino acids, glycylserine, or other serine dipeptides for growth of a serine-requiring mutant of *Escherichia coli* (strain K-12). The authors believe that if the peptide linkage of serylglycine serves only to prevent the rapid deamination of serine, the other peptides of this amino acid should be similarly effective. Also of interest is the observation of Mueller & Miller (260) that a pancreatic digest of casein is required for tetanus toxin production by *Clostridium tetani* although a complete acid hydrolysate supports abundant growth. Miller (261) has further shown that peptides of histidine are effective in supporting toxin production. However, these peptides may contain nonprotein components and presumably are utilized only after hydrolysis.

Several workers have found that whole proteins may be interconverted without the apparent liberation of free amino acids. Thus the early work of Yuile *et al.* (262) showed that plasma proteins labeled with lysine-ϵ-C^{14} are converted to tissue proteins in the dog with very small loss of C^{14} in the urine or expired air. The authors favor the idea of utilization of plasma proteins after partial breakdown to peptides within the cell. Similar results were reported for the rat by Babson & Winnick (263) and Gavrilova (264). These workers found that the radioactivity of plasma proteins containing labeled leucine or methionine is incorporated into tissue protein but cannot be diluted by concurrent administration of the respective nonradioactive amino acid. The failure to observe this dilution is interpreted as indicating that the label in the injected protein did not pass through the free amino acid stage prior to the synthesis of new protein. According to the above workers and also to Busch *et al.* (265) and to Bauer and co-workers (266) such uptake of plasma protein is especially efficient in tumors. In the studies of Yuile *et al.* (262) it was assumed that if amino acids were formed after intracellular protein hydrolysis, some would escape from the cell. However, any labeled amino acid in such protein, upon liberation, would be located in a medium containing a normal balance of other amino acids and thus may enter cellu-

lar protein without significant loss to the circulation. Such is the explanation by Maurer & Müller (267) for the interconversion of serum albumin to globulin in the rat. The failure to demonstrate a dilution of label following administration of nonlabeled amino acid (263, 264) may be attributable to the inability to maintain a large intracellular pool of nonlabeled amino acid during the period of protein synthesis.

Studies by Francis & Winnick (268) and Winnick & Winnick (269) have indicated that labeled proteins of embryo extracts are incorporated into the cell protein of embryonic chick tissue cultures without complete breakdown to amino acids. However, it is not clear just what percentage of the proteins is to be considered as new cell proteins, for absorbed proteins maintain their immunological specificity (270). Also the observation by Francis & Winnick (268), that concentrations of dinitrophenol which inhibit growth and the incorporation of radioactive amino acids do not influence protein transfer, may indicate that little conversion to new cell protein need occur. This view is supported by the observation of Eagle (204, 205) that dialyzed serum did not satisfy the nitrogen requirements in his tissue culture preparations.

In contrast to the aforementioned utilization of serum proteins, negative evidence has been reported with regard to their utilization in the synthesis of goat milk proteins [Askonas, Campbell & Work (271)] and the proteins of rat pancreatic juice [Junqueira, Hirsch & Rothschild (272)].

Evidence that free peptides are not formed during protein synthesis has been supplied by Halvorson, Spiegelman & Hinman (273). These workers reported that tryptazan, a potent tryptophan antagonist, inhibited the induction of maltase and synthesis of protein in yeast without demonstrable accumulation of peptides. Similar results had been reported earlier with the use of phenylalanine analogues [Halvorson & Spiegelman (274)].

Flavin & Anfinsen (275) found that one of the 11 dipeptides of cysteine isolated from ovalbumin hydrolysates was cysteinylglycine. However, incubation of oviduct mince with glutathione, doubly labeled in the cysteinylglycine moiety, did not result in preferential labeling of the same sequence in ovalbumin. The results do not support any specific role of glutathione in the biosynthesis of protein cysteinylglycine. It would be of interest to learn whether any multiple-labeled peptide, possibly a biologically labeled peptide growth factor, could retain its label upon incorporation into protein.

The synthesis of proteins may take place either by a mechanism in which the component amino acids combine simultaneously on a sequence-determining template or by a stepwise process. While the latter process may involve peptides, it is not necessary that they be free, for they may also be bound as intermediary complexes in close proximity to the protein synthetic mechanism. Such intermediates may be demonstrable if they equilibrate at different rates with their constituent amino acids. In attempts to distinguish between the simultaneous and stepwise processes, Anfinsen and co-workers have determined the specific activity of various individual amino acids in

different peptide fractions of several characterized proteins which were isolated from tissues incubated with radioactive amino acids. Within the period covered by this review Flavin & Anfinsen (275) found that glycine or serine had different specific activities when isolated from peptides derived from different portions of ovalbumin which had been obtained from oviduct mince incubated with glycine-1-C^{14}. Similarly, Vaughan & Anfinsen (276) found that glycine, serine, and phenylalanine had unequal distributions when isolated from different peptide fractions either of insulin or ribonuclease obtained after incubation of calf pancreas with radioactive glycine or phenylalanine. It is of interest that the specific activity of the glycine was different in chains A and B of insulin and was also nonuniform in two locations in chain B. These authors recognize the possibility that unequal distribution may be attributable to unequal exchange reaction rates at various portions of the protein molecule (see page 386).

The studies on unequal labeling have stimulated much thought and effort to generalize and explain this phenomenon. Askonas *et al.* (277) could not find an unequal distribution of several amino acids in casein or lactalbumin obtained from a goat 3 to 4 hr. after injection of the radioactive amino acids. Heimberg & Velick (278), using a different approach, crystallized aldolase and phosphorylase from muscles of rabbits injected at intervals during a 24-hr. period with radioactive glycine, alanine, lysine, and phenylalanine. The ratios of the specific activities of the amino acids isolated from these two enzymes were the same for each amino acid. This may be interpreted as an indication of equal labeling of these amino acids in each enzyme. Since it had been demonstrated that the inequality of labeling diminishes with longer times of incubation (279), Simpson (280) repeated his earlier work with Velick (281), now waiting only one-half hour between injection of the amino acids and isolation of aldolase and glyceraldehyde-3-phosphate dehydrogenase. The pattern of radioactivity, nevertheless, indicated uniform labeling in each enzyme.

Steinberg (282) has proposed that the failure to demonstrate unequal labeling in experiments *in vivo* may be attributable to the rapidity of equilibration of intermediates with the amino acid pool. The rate of equilibration may be diminished under the less optimal conditions for protein synthesis brought on under conditions *in vitro*. It is perhaps also of interest that all of the proteins shown to have unequal labeling are formed for secretion.

To explain unequal labeling, Dalgliesh (283) suggested a template mechanism in which the completed end of a protein is released while the rest of the molecule is being formed. If the time for synthesis were sufficiently long and if the specific activities of the constituents of the amino acid pool varied, then the residues formed near the initial end would reflect the earlier specific activities. However, at least in the case of dicarboxylic amino acids used for labeling ovalbumin, such a change in amino acid specific activity did not occur [Peters (284)].

Precursors of High Molecular Weight in Protein Synthesis

The disease known as multiple myeloma promotes the synthesis of a number of diverse proteins, some apparently new to the organism. One of these, the Bence-Jones protein, is excreted in the urine as a waste product. Highly purified preparations of this protein have been shown by Deutsch, Kratochvil & Reif (285) to have immunological similarities to normal serum globulins. Tracer studies by Putnam & Hardy (286, 287) indicate that the Bence-Jones protein is synthesized rapidly from the amino acid pool rather than from any plasma or tissue protein precursor. It does not appear to be a precursor for the anomalous serum globulins formed during this disease. Putnam (288) considers that it may be an incomplete protein, perhaps an unfinished precursor of normal globulins or an abortive product of the deranged metabolism of serum protein synthesis.

The soluble proteins which react with virus anti-serum and accumulate in the tobacco leaves following infection with tobacco mosaic virus have been shown by Van Rysselberge & Jeener (289) to behave as the virus protein precursor. After exposure of the tobacco leaves to $C^{14}O_2$, the soluble antigens had a higher specific activity than the virus protein but did not increase in amount during the experiment, as did the virus. According to Delwiche & co-workers (290), N^{15}-labeled ammonia was incorporated into the soluble antigens at essentially the same rate as into the virus, suggesting that although the isolated antigen may not be a precursor, this material in nascent form at the synthetic site in the tobacco cell may be. This is in accord with the observation of Commoner & Rodenberg (291) that the soluble antigens appear only 220 hr. after the infection, coinciding with the depletion of the nucleic acid store involved in virus synthesis. The picture appears more complicated because at least three nonvirus proteins which cross react with virus-antiserum have been isolated [Commoner & Yamada (292)].

Peters (293) had earlier demonstrated that upon incubation of chick liver slices with radioactive bicarbonate or glycine, an induction period of approximately 20 min. was required before any incorporation of radioactivity into serum albumin occurred. Since no such lag period was observed in the incorporation of C^{14} into total liver proteins, it appears to be attributable to the formation of a serum albumin precursor. This induction period has been studied under conditions *in vivo* by Green & Anker (294). With rabbits the lag period after intravenous injection of glycine was 25 min. at 39.5°C. and 2.5 hr. at 24°C. Similar results were obtained with serum of turtles (*Pseudomys elegans*) and for incorporation of glycine into hemocyanin of the horseshoe crab (*Limulus polyphemus*). The lag period was independent of isotope concentration, the amino acid injected, starvation, simultaneous administration of an amino acid mixture, or amino acid antagonists. It appears to be related to the process of synthesis, although the release of serum protein into the circulation may be involved. A similar lag period

was observed by Junqueira *et al.* (272) for the incorporation of radioactive glycine into the protein of rat pancreatic juice.

Halvorson & Jackson (295) have noticed that as the formation of the induced enzyme, α-glucosidase, progresses in yeast, the extent of inhibition of its synthesis by the subsequent addition of tryptazan decreases. This tryptophan antagonist, however, still completely prevented the utilization of the amino acid pool for protein synthesis. The results are interpreted as indicating the transformation of a precursor into the enzyme without further utilization of amino acids; during the earlier stages of induction the antagonist prevented the formation of the precursor itself. If such precursor material is present in the unadapted cells, it must exist in minute quantities, for *E. coli*, initially labeled with C^{14} or S^{35}, did not produce labeled β-galactosidase shortly after induction [Rotman & Spiegelman (296); Hogness, Cohn & Monod (297)]. However, some precursor may be demonstrable if induction occurs in the absence of cell division [Rickenberg & Lester (298)]. Loftfield (299) claims that rats induced to form ferritin by iron injection derive most of the new protein from amino acids. Similar conclusions with regard to antibody production have been made by Green & Anker (300), indicating that any intermediates for these proteins must be present in small quantity.

Allfrey, Daly & Mirsky (301) isolated a protein fraction from a ribonuclease digest of microsomes from mouse pancreas which attained the highest specific activity of any protein from this tissue after injection of labeled glycine. The concentration of isotope in this protein increased during the synthesis of pancreatic digestive enzymes, indicating a possible precursor relationship. In a similar vein, Littlefield and co-workers (302) obtained a ribonucleoprotein by treating rat liver microsomes with sodium deoxycholate. After injection of a radioactive amino acid, labeling of this protein reached a maximal value within a few minutes and then decreased, while the radioactivity of other liver proteins progressively increased.

Evidence for what appears to be a late intermediate stage in the production of β-galactosidase by *E. coli* was presented by Bonner (303). During adaptation, the ratio of enzyme activity in the cell extract to the enzyme activity in the whole cells is high; an unavailability of the intracellular enzyme is indicated. This cannot be attributed to poor permeability of substrate into the cell, for the ratio decreases rapidly during de-adaptation. The fact that there is more masked enzyme during adaptation than during de-adaptation suggests that much of the enzyme may be bound to the enzyme-forming system during adaptation. A similar observation was made by Rotman (304). The possibility must also be considered that the enzyme is bound to particles which are not involved in enzyme synthesis.

Nucleic Acids and Protein Synthesis

The cytochemical and biochemical correlative studies between nucleic acid content of cells and protein synthesis have recently been reviewed by

Brachet (305). Further work has shown that the action of ribonuclease inhibits the incorporation *in vitro* of radioactive amino acids into cells and their particulate matter: live amoebae (306), onion root tips (307), protoplasts of *Micrococcus lysodeikticus* (308, 309), microsomes of liver (244), and pancreas (301). The inhibition of the protein-forming system in microsomes by ribonuclease action may be specific, for Novikoff, Ryan & Podber (310) have shown that the activities of several microsomal enzymes are unaffected by removal of RNA. Ribonuclease action can also prevent enzyme induction in protoplasts of *Bacillus megatherium* (311) and enzyme synthesis by a solubilized preparation from pancreas (312).

Of particular interest has been the reconstruction of protein-forming systems by the addition of nucleic acid or its hydrolyzed products to broken cell preparations. Thus Gale & Folkes (313) [see also Gale (314)] reported that *Staphylococcus aureus* DNA and RNA, or its ribonuclease digest, could support the incorporation of radioactive glutamate into protein when added to disrupted cells which had been depleted of their nucleic acid. Nucleic acids obtained from other species were ineffective. These workers further demonstrated (315) that the formation of enzymes by their preparation depended upon addition of RNA at the early stages of nucleic acid depletion, but at a later stage DNA was required. For formation of catalase, a constitutive enzyme, only RNA obtained from *S. aureus* was required, and a purine pyrimidine mixture was ineffective. For formation of the induced enzyme, β-galactosidase, a purine-pyrimidine mixture was required, and RNA or DNA of either adapted or unadapted cells was ineffective. This is in accord with the concept that synthesis of RNA is linked to the formation of adaptive enzymes. However, Reiner & Goodman (316) found that RNA or its ribonuclease-digested material, which had been obtained from gluconate-adapted *E. coli*, was specific for transferring adaptation to cells grown on lactate. Of major importance has been the observation of Gale & Folkes (317) that various fractions associated with di- and trinucleotides from ribonuclease digests of RNA from *S. aureus* could catalyze the exchange of amino acids with corresponding residues in proteins of disrupted cells. Certain fractions were somewhat specific for incorporation of individual amino acids. Thus an approach to a nucleic acid template may be beginning to appear. An experimentally verifiable template structure, wherein overlapping of the nucleic acid bases which determine amino acid location would limit the number of possible sequences in protein, has been postulated by Gamow (318, 319).

Co-synthesis of RNA and protein.—Much experimental work points to the possibility that concurrent RNA synthesis is required for protein synthesis. Pardee (320) has shown that purine- and pyrimidine-requiring strains of *E. coli* cease to form induced enzymes soon after exhaustion of limiting amounts of the required base. The RNA formed during early stages of induction is not used. The bulk of the cellular RNA can supply materials for this purpose only after energy depletion of the cells and then the enzyme is synthesized in the absence of cell division [Pardee (321); see also Ricken-

berg & Lester (298)]. Thus if the intracellular role of ribonuclease is to remove RNA from newly synthesized protein, as suggested by Roth (322), it does not at the same time break it down to units that can be used for resynthesis. After inducing β-galactosidase synthesis in *E. coli*, Spiegelman and co-workers (323) added the uracil antagonist, 5-hydroxyuridine, at a level which had no effect on growth; they observed a temporary cessation of enzyme synthesis. Since the enzyme-forming system was present in the early stages of induction, only the rate of enzyme synthesis should be altered if co-synthesis of RNA were not required. In a similar study Creaser (324) found that the purine antagonist, 8-azaguanine, by being incorporated into the new RNA, blocked the formation of β-galactosidase in *S. aureus*. With yeast, which unlike *E. coli* has a nucleotide pool, previously initiated α-glucosidase production could be stopped by depleting the pool through the stimulation of synthesis of other protein [Spiegelman, Halvorson & Ben-Ishai (323)]. This experiment may indicate a co-synthesis requirement of RNA and enzyme, but the results can also be interpreted by assuming a breakdown of the RNA enzyme-forming system during depletion of the nucleotide pool. In view of the possibility that the observed requirement for co-synthesis of RNA in formation of induced enzymes is inherent to the instability of the RNA involved in the production of such enzymes, it is of interest to observe the results obtained with other protein-forming systems.

If one inhibitor is found which inhibits the synthesis of either RNA or protein without inhibiting the other, then it would appear that their co-synthesis is not obligatory. Thus chloramphenicol has been shown to inhibit net protein synthesis in *S. aureus* but not net nucleic acid synthesis [Gale & Fokes (325)], and similar effects have been shown in *E. coli* with the use of radioactive glycine [Wisseman *et al.* (326)]. Although protein formation is inhibited by chloramphenicol, free amino acids must be present to permit RNA synthesis, thus indicating the possible formation and breakdown of associated protein (327). A reverse phenomenon has been demonstrated by McQuillen (328) in the protoplasts of *B. megatherium;* uranyl chloride inhibited the incorporation of radioactive glycine into nucleic acids but not into protein. Abrams (329) had previously shown that x-irradiation of rats or rabbits produced the above inhibition pattern of glycine incorporation into several tissues. The locus of inhibition, however, may be at one of the many enzymatic steps involved in purine formation from glycine and may not involve RNA synthesis from existing nucleotides.

Stimulatory effects should be reflected in both protein and RNA formation if their co-synthesis is obligatory. Reddi (330) had reported that methionine stimulates net protein synthesis in *Pseudomonas hydrophila* and also increases P^{32} incorporation into RNA adenylic, guanylic, and cytidylic acids but not into uridylic acid. Stimulation of pigeon pancreas secretion with carbamyl choline is followed by an increased incorporation *in vivo* of glycine into protein, which is paralleled by an increased incorporation of P^{32} into

RNA [Fernandes & Junquiera (331)]. However, Hokin & Hokin reported that the increased amylase synthesis by pancreas slices caused by the presence of an amino acid mixture was not accompanied by an increase in the level of RNA (332) or in the incorporation of P^{32} into its nucleotides (333). In view of the conflicting evidence, it is difficult at present to ascertain whether co-synthesis of RNA is a general property of protein synthesis.

Role of the nucleus and DNA in protein synthesis.—Although DNA appears to be involved in amino acid incorporation and enzyme synthesis in particulate fractions from *S. aureus* (313, 314, 315), inhibition of DNA synthesis by ultraviolet light (295, 334), mustard gas (320, 335), or thymine deficiency (336) does not stop bacterial enzyme synthesis. Klein & Forssberg (337) have dissociated DNA synthesis from the synthesis of RNA and protein in the Ehrlich ascites cell by use of x-irradiation. Vanderhaeghe (338) has reported that growth and net synthesis of protein can occur in enucleated halves of the algae, *Acetabularia mediterranea*, although with *Amoeba proteus*, both absorption and incorporation of methionine-S^{35} are reduced in such enucleated halves [Mazia & Prescott (339)]. The latter authors indicate that the nuclei of amoebae contain much RNA and appear to be an independent site of protein synthesis.

Allfrey and co-workers (340, 341) have shown that isolated calf thymus nuclei incorporate amino acids into their protein by a synthetic mechanism. The nuclear protein of several adult mouse tissues is metabolically active (342). With the use of autoradiographic techniques, Sirlin (343) has demonstrated an uptake of methionine-S^{35} by the nuclei of *Triturus alpestris*.

Protein Turnover and Amino Acid Exchange

Following their early classical experiments on the incorporation of labeled leucine into various adult rat tissues, Schoenheimer, Ratner & Rittenberg (344) concluded, "There are two general reactions possible which might lead to amino acid replacement, (*a*) complete breakdown of the protein into its units followed by resynthesis or (*b*) only partial replacement of units." Recently some questions have been raised concerning the existence of the first mechanism. The second reaction has been demonstrated and its general significance in isotopic studies with proteins suggested.

In studies with *E. coli*, Hogness, Cohn & Monod (297) demonstrated that bacteria containing S^{35}-labeled protein contributed no radioactivity to β-galactosidase which was subsequently induced and isolated. Conversely, labeled β-galactosidase lost no measurable radioactivity in growing cultures, either in the absence or presence of the inducer. In an extension of these observations to the loss of proteins by mammalian tissues, the authors suggest that such losses may be limited to extracellular proteins and that intracellular degradation does not occur. Rotman & Spiegelman (296) have criticized this interpretation; they indicate that protein breakdown rates characteristic of mammalian tissues would not be observable during the short time period employed by Hogness *et al.* When the growth period of

E. coli was extended to 30 hr. Podolsky (345) found that at least 8 per cent of the labeled arginine in protein was lost. Moreover, Koch & Levy (346) demonstrated that while the doubling time of *E. coli* is 1 hr., the minimum half time for degradation of proteins is 30 days.

Direct evidence for the intracellular degradation of proteins in yeast has been presented by Halvorson and co-workers (225, 347, 348), who found that, although during the early stages of nitrogen starvation the free amino acid pool is diminished to 10 per cent of normal, the organism still possesses a marked capacity to synthesize α-glucosidase. That this synthesis involves utilization of amino acids, as it does in the unstarved cell, is evident from the competitive inhibition observed with amino acid analogues. Furthermore, it was shown that under conditions of limiting energy supply an increase in the free amino acid pool can take place at the expense of protein. This phenomenon, termed "internal replenishment," decreases with prolonged starvation because the more stable proteins of the cell are formed at the expense of the labile components. An internal replenishment mechanism has also been reported in *Micrococcus lysodeikticus* (349).

Penn, Mandeles & Anker (350) noticed that when a mixture of radioactive amino acids was injected into a rabbit and the serum albumin isolated over a period of one month, C^{14} loss from the various amino acids in the protein proceeded at different rates. They stress that the rate of loss of isotope may depend upon equilibration with labeled amino acids in the other proteins of the animal. Rates of serum protein turnover which account for such equilibration have been calculated by Niklas *et al.* (351, 352, 353).

The incorporation of an amino acid into protein by exchange can occur by displacement of a corresponding residue in either a complete protein or in one at an intermediate stage of synthesis. Evidence for this phenomenon may be either implied, as in the demonstration that labeled amino acids are incorporated under conditions wherein no protein can be synthesized, or may be direct, as the displacement of a labeled amino acid residue in a protein by the corresponding nonlabeled amino acid in the medium.

Both implied and direct evidence has been furnished by Gale & Folkes with the use of intact (354, 355) and broken cell (313) preparations of *S. aureus*, respectively. Labeled glutamic acid can be incorporated into protein by this organism when it is the sole amino acid in a medium containing glucose (condition 1) or when it is present with glucose and a full complement of amino acids (condition 2). Under condition 1 incorporation decreases with time and takes place at a slower rate than under condition 2. The incorporation under condition 2 continues linearly for several hours and can be correlated with a net increase in protein. Several lines of evidence indicate that incorporation under condition 1 does not represent synthesis of some proteins coupled with the breakdown of others: (*a*) omission of aspartic acid from an otherwise complete mixture prevents net protein synthesis but increases the initial rate of glutamic acid incorporation; (*b*) incorporation of labeled glutamic acid under condition 1 is much

less sensitive to inhibition by chloramphenicol or chlortetracycline (aureomycin) but is more sensitive to inhibition by penicillin or bacitracin than under condition 2; (*c*) the incorporation of different amino acids is inhibited to various extents by the antibiotics. Even under condition 2 incorporation may not be identical with protein synthesis, for here *p*-chlorophenylalanine inhibits phenylalanine incorporation and the net increase of glutamic acid in protein but has no significant effect upon the incorporation of labeled glutamic acid. In studies with a disrupted cell preparation, Gale & Folkes (313) found that glutamic acid can be incorporated into protein in the presence of an energy source and that it can be released from the protein by subsequent incubation with unlabeled glutamic acid and an energy source. These authors further demonstrated that protein synthesis and glutamic acid incorporation can be inhibited by depletion of RNA and restored by RNA addition. In the presence of a complete amino acid mixture, an energy source, and RNA from *S. aureus* net protein synthesis occurred in the reconstituted system, but the addition of RNA did not result in a proportional rise in incorporation of all labeled amino acids. In particular, the presence or absence of RNA had no effect upon the incorporation of alanine. These differences between protein synthesis and amino acid incorporation in *S. aureus* imply that much work utilizing amino acid incorporation as a measure of protein synthesis may require reevaluation. Nisman, Hirsch & Marmur (356) have confirmed the work of Gale & Folkes by demonstrating the replacement of incorporated amino acids from protein of *E. coli* preparations. A small energy-dependent release of amino acids from protein was demonstrated for rat liver slices by Simpson (357). However, amino acids incorporated into protein of rat liver microsomes (302) or calf thymus nuclei (341) were not exchangeable.

It should be noted that the release of incorporated glutamate occurred only from the insoluble protein of the disrupted cells (313) which contained the particulate matter involved in synthesis. Gale (314) has speculated that exchange may occur only in the nucleoprotein fraction.

Mammalian tissues have long been known to carry out the incorporation of individual amino acids *in vitro* without demonstrable increase in protein even in the presence of a complete amino acid mixture. Such incorporation of amino acids into the proteins of Ehrlich ascites cells appeared to be an exchange process, for phenylalanine antagonists were specific in their inhibition of phenylalanine incorporation [Rabinovitz, Olson & Greenberg (247)]. A similar result was obtained with ethionine which only inhibited methionine incorporation. However, a balance study with radioactive ethionine indicated that it was incorporated in place of methionine (358). Such incorporation of amino acid analogues into protein, also observed with *Lactobacillus arabinosus* [Baker, Johnson & Fox (359)] and *Tetrahymena pyriformis* [Gross & Tarver (360)], renders difficult the use of amino acid analogues for studying the mechanism of amino acid incorporation. Although some amino acid analogues substitute for their corresponding

metabolites, others have been found which act as inhibitors of protein synthesis in the Ehrlich ascites cell. Thus O-methyl-threonine inhibited the incorporation of its metabolite, isoleucine, competitively, but its inhibition of leucine incorporation was noncompetitive and was relieved by isoleucine [Rabinovitz, Olson & Greenberg (361)]. Also, methionine sulfoximine inhibited the incorporation of several amino acids noncompetitively, and this inhibition was relieved by glutamine [Rabinovitz & Olson (362)]. The extent of these inhibitions indicates that at least half of the incorporative activity of the Ehrlich ascites cell must be attributable to protein synthesis.

Control of Protein Metabolism

Nature of the adaptive response.—The earlier literature concerning the role of the inducer in the synthesis of enzymes has been summarized by Spiegelman & Halvorson (363). These workers favor a catalytic role for the inducer because α-methylglucoside has a 250-fold greater affinity for whatever structure it combines with as an inducer than it has for the yeast enzyme induced α-glucosidase. Similar results have been obtained for the induction of lysine decarboxylase in *Bacterium cadaveris* under aerobic conditions [Mendelstam (364)]. In contrast, Dubnoff (365) reactivated several inactive bacterial enzymes with a vitamin B_{12} system and demonstrated that some enzymes may become rapidly inactivated upon removal of the activator unless substrate is added. He believes that the inducer's role may be explained entirely by the stabilization of the enzyme as enzyme-substrate complex, and points out that the stabilization concept accounts for the demonstration of the enzymatically inactive but antigenically similar Pz protein found by Cohn & Torriani (366) as a companion to β-galactosidase. The enzyme-stabilization concept may be considered to be supported by the work of Cohen & Rickenberg (367) who demonstrated that *E. coli* fixed very little of the nonsubstrate inducer, thiomethyl-β-D-galactoside, before induction, thus indicating the absence of another binding site where the inducer may act as catalyst for enzyme formation. The enzyme-stabilization concept, however, fails to account for the profound influence of the inducer upon RNA synthesis, which was demonstrated by Gale & Folkes (315) as an increased uracil incorporation into RNA during the preinductive lag period. Such data might place the inducer as a gear in the enzyme synthetic mechanism rather than as a protective coating for the finished product.

Similarly, Fineberg & Greenberg (368) found that in the induction of ferritin production in guinea pigs by iron, there was an acceleration of apoferritin synthesis, not merely stabilization by bound iron. This evidence supports a catalytic role for the inducer at the site of apoprotein synthesis but allows an alternative hypothesis, as pointed out by the authors (369): inhibition by apoferritin of its own synthesis, the inhibition being relieved in the conversion of apoferritin to ferritin by iron. Evidence for the latter concept in the case of catalase has been put forth by Chantrenne (370),

who demonstrated an increased enzyme formation in yeast aerated in the presence of azide, a catalase inhibitor.

Hormonal control of protein synthesis.—The increase in the liver tryptophan peroxidase activity following cortisone injection has been studied by Knox & Auerbach (371) who suggested that it may be attributable to the higher free amino acid content in the blood following this treatment. However, the increases observed after hormone injection are not paralleled by a corresponding increase in circulating tryptophan (372). The rise in enzyme activity is reproducible and has been suggested as an assay method for corticoid hormones [Thomson & Mikuta (373)]. Geschwind & Li (374) reported a variety of hormonal effects upon formation of liver tryptophan peroxidase. The level of other enzymes is also increased by cortical hormones, as for example, liver glucose-6-phosphatase [Weber *et al.* (375)].

The mechanism of these effects by the cortical hormones may be explained by the observations of Roberts (376) who presented evidence that these hormones labilize tissue protein which can then be used for anabolism or catabolism, depending upon the tissue requirements for protein at the moment. Thus, Lawrence & MacVicar (377) observed a decreased utilization of dietary nitrogen for protein synthesis in the rat after cortisone treatment. Also, Ulrich, Tarver & Li (378) reported an increased rate of serum albumin degradation by rats given ACTH as compared to the untreated hypophysectomized rat. Cortisone appears to "depolymerize" the RNA of liver mitochondria and microsomes [Lowe & Williams (379)] without affecting the P:O ratio obtained with the mitochondria [Lowe & Lehninger (380)] or the *in vivo* incorporative activity for labeled amino acids by the microsomes [Mirsky *et al.* (381)]. Present knowledge of the nature of hormonal control of amino acid metabolism has been summarized by Russell (382). Bartlett (383) has discussed the promotion of nitrogen storage by growth hormone in the adult dog.

Protein metabolism in pathological states.—Burke & Miller (384) have observed a twofold increase in incorporation of labeled lysine into liver proteins of perfused precancerous or cirrhotic rat liver, with no effect on incorporation into serum albumin. Similarly, human patients with advanced portal cirrhosis have low concentrations of plasma albumin but may maintain a normal rate of albumin synthesis and catabolism [Volwiler *et al.* (385)].

Allison *et al.* (386) report that when the casein content of the diet was increased, a longer induction period was required for the transplanted R1 rat sarcoma; however, the level of dietary protein did not influence subsequent growth of the tumor. In tumor-bearing rats, the content of liver protein and enzymes are maintained when the animals are on a protein-free diet (387). Babson & Winnick (263) and Greenlees & LePage (388) have demonstrated that tumors can lose labeled amino acids from their protein during growth, thus indicating that a tumor is not completely a "nitrogen trap." In several

transplant generations after primary tumors a greater affinity is shown by the tumor for retention of labeled protein during fasting (388).

Injection of rabbit anti-rat kidney serum into rats produces an experimental nephrosis which results in a daily excretion of more than twice the total circulating albumin and a replacement rate three times normal [Drabkin & Marsh (389)]. Niklas and co-workers, however, did not find increased albumin synthesis in similarly treated rabbits (390), nor in bled rats (391).

Cysteine appears to be the limiting amino acid for the healing of experimental skin wounds in rats. Livers of injured rats convert methionine-S^{35} to cysteine more rapidly than do the livers of normal animals [Fromm (392); Williamson & Fromm (393)].

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II. Protein Metabolism

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WATER-SOLUBLE VITAMINS, PART I[1]

(Vitamin B_{12}, Folic Acid, Choline, and Para-Aminobenzoic Acid)

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This review surveys the literature available to the authors from about December 15, 1954 to December 15, 1955. Reference is made only to those clinical papers which bear directly on the biochemical topics discussed.

Vitamin B_{12} and Related Substances

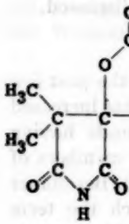
Nomenclature in this field has grown more involved during the past few years as new members of the B_{12} group have been uncovered. It has increased in complexity with the biosynthetic preparation of compounds having "unnatural" nucleotide bases. The known naturally occurring members of the group have been recently correlated and catalogued (1, 2, 3). Bernhauer & Friedrich (2) suggested a system of nomenclature in which the term "cobalamin" is used in conjunction with the nucleotide base. Thus, B_{12} is referred to as 5,6-dimethylbenzimidazole-cobalamin; pseudo B_{12}, adenine-cobalamin; Factor A, 2-methyladenine-cobalamin, etc. Factor B (B_{12} minus the nucleotide) is referred to as etiocobalamin. Such a system has the merit of providing a ready means of reference to the potentially infinite number of biosynthetic B_{12}-like compounds. It would also make unnecessary many current trivial names and could eventually be extended to make provision for those compounds known to have a common nucleotide base but differing in other respects. This suggested system of nomenclature has not been used in this article since the reviewers feel that the implied change in the meaning of the term, cobalamin, may be confusing to the reader.

In last year's review Briggs & Daft (4) commented on the little progress which had been made in the determination of so complex a structure as that of B_{12}. Yet six months later contributions from several laboratories permitted the proposal of a complete structural formula (5, 6, 7).

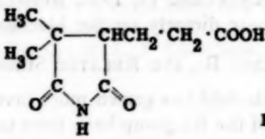
Structure of B_{12}.—Kuehl, Shunk & Folkers (8, 9) isolated two new cystalline compounds, $C_9H_{11}NO_4$(I) and $C_9H_{13}NO_4$(II), from oxidized (sodium chromate in acetic acid) acid-hydrolyzed B_{12}. The imide character of the weakly acidic I and the presence of a γ-lactone group were indicated from

[1] The following abbreviations are used in this chapter: 4-APGA for 4-aminopteroylglutamic acid; ATP for adenosinetriphosphate; B_{12} for vitamin B_{12}; pseudo B_{12} for pseudovitamin B_{12}; CF for citrovorum factor; CoA for coenzyme A; DNA for deoxyribonucleic acid; DPN for diphosphopyridine nucleotide; FA for folic acids (the group of natural compounds which promote the growth of *Lactobacillus casei* or *Streptococcus faecalis*); IF for intrinsic factor; PABA for *p*-aminobenzoic acid; PGA for pteroylglutamic acid; $PGAH_4$ for tetrahydropteroylglutamic acid; RNA for ribonucleic acid.

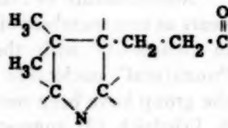
infrared absorption properties. It yielded on hydrolysis a lactone dicarboxylic acid. Succinic and isobutyric acids were formed on alkaline fusion. Distillation with zinc dust produced a base reacting as a pyrrole. Of two possibilities, the correct structure was proved by synthesis to be DL-3,3-dimethyl-2,5-dioxo-4-hydroxypyrrolidine-4-propionic acid lactone (I). The lactone dicarboxylic acid mentioned above was synthesized by hydrolyzing the cyanohydrin diethyl ester of 2,2-dimethyl-3-oxo-adipic acid. The pyrrolidine (I) was obtained on pyrolysis of the ammonium salt.



I



II



III

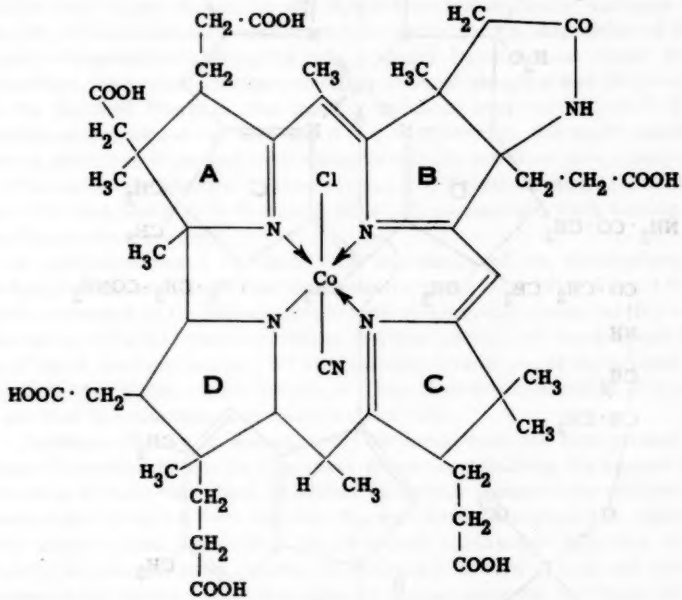
The imide II on hydrolysis formed a tricarboxylic acid which was found to be identical with the known 2,2-dimethyl-3-carboxy-adipic acid. DL-3,3-Dimethyl-2,5-dioxo-4-hydroxypyrrolidine-4-propionic acid (II) was obtained on pyrolysis of the tricarboxylic acid following treatment with one mole of ammonia.

These observations proved the presence of the structural moiety III in the B_{12} molecule. Evidence that structure III occurs in B_{12} in amide form was furnished by Kuehl and co-workers (10). They isolated a crystalline compound from the chromic acid oxidation of unhydrolyzed B_{12} identical with the synthetic amide of II. The imide II was subsequently obtained by chromic oxidation of the crystalline cobalt-containing hexacarboxylic acid degradation product of B_{12} by Bonnett *et al.* (6), offering confirmatory evidence for the presence of this succinimide-type grouping in the pigmented nucleus of B_{12}. They also isolated oxamic acid, HOOC·CONH$_2$, on oxidation of the hexacarboxylic acid with hydrogen peroxide.

Schmid, Ebnöther & Karrer (11) had previously obtained oxalic, succinic, methylsuccinic, dimethylmalonic acid, and several unidentified acids by permanganate oxidation of acid-hydrolyzed B_{12}. One of the latter, Acid B, was recognized by Garbers, Schmid & Karrer (12) as DL-2,2-dimethyl-3-carboxy-adipic acid identical with the acid obtained by Kuehl *et al.* (8) on hydrolysis of the succinimide II.

Cannon, Johnson & Todd (13) in continuing earlier hydrolysis studies (13a) isolated a red crystalline hexacarboxylic acid following treatment of B_{12} with 30 per cent sodium hydroxide at 150°C. Amorphous tetra- and pentacarboxylic acids were encountered in lesser yield. All were nucleotide-

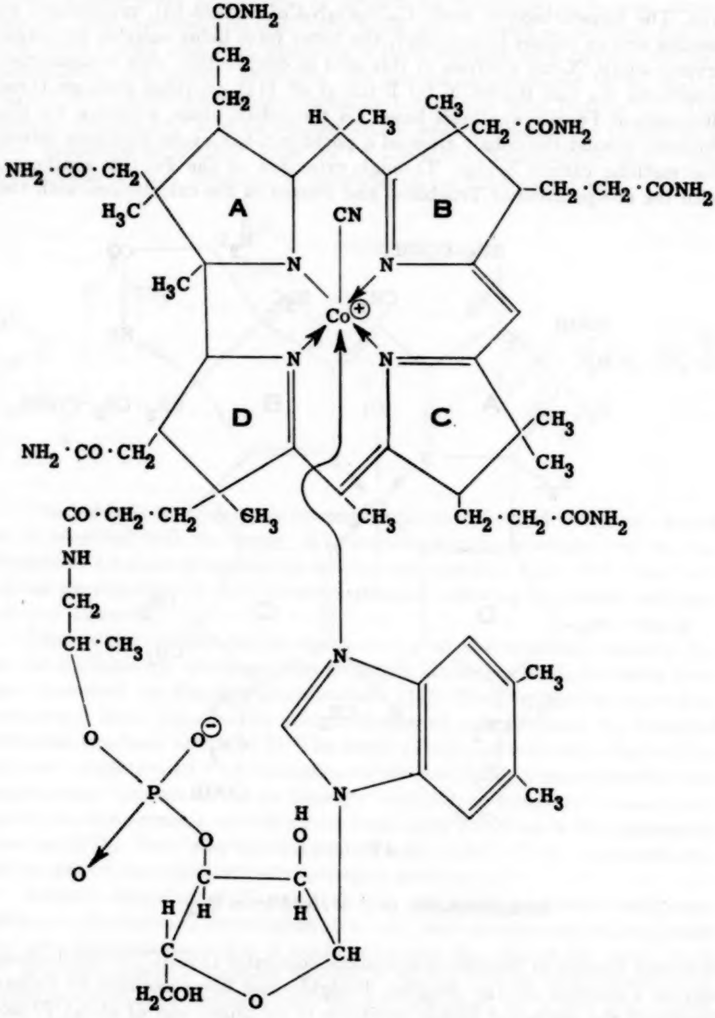
free. The hexacarboxylic acid, $C_{46}H_{60}O_{13}N_6CoCl \cdot 2H_2O$ (6), crystallized as needles and as prisms (dimorphic), the latter form being suitable for single crystal study. X-ray analysis of this acid in conjunction with comparative results on B_{12} and B_{12}-SeCN by Brink *et al.* (14) provided through three dimensional Fourier synthesis based on the cobalt atom, evidence for the presence around the cobalt atom of a porphyrin-like cyclic structure minus one methine carbon bridge. Through extension of the Fourier syntheses, with the co-operation of Trueblood and Prosen in the calculations with the



IV

Hexacarboxylic acid derived from B_{12}

National Bureau of Standards automatic computer (SWAC) at the University of California at Los Angeles, Hodgkin and her associates at Oxford identified the projected atomic positions of 62 atoms out of about 70 not counting hydrogen. From crystal structure findings in conjunction with chemical evidence Hodgkin and her colleagues (5) and Todd, Johnson, Smith and their associates (6) proposed the structure IV for the hexacarboxylic acid and the structure V for B_{12} itself. The five-membered lactam ring fused to ring B in the hexacarboxylic acid presumably arises from closure



V

Vitamin B_{12} $C_{63}H_{90}O_{14}N_{14}PCo$

of the ring following hydroxylation of ring B during hydrolysis of B_{12} in strong alkali. The optically inactive lactone succinimide I of the Merck group must then have been formed by B-hydroxylation of ring C. A number of features of these structures are admittedly not proved, but the proposed structures are in agreement with all presently available physical and chemical evidence. Two of the less certain details are the number and position of the double bonds and the nature of the single substituent groups. Todd *et al.* propose an interesting biogenetic scheme for the formation of the B_{12} chromophore from porphobilinogen. Kamper & Hodgkin (15) extended their x-ray studies to the 5,6-dichlorobenzimidazole analogue of B_{12} (16, 17) and obtained confirmatory evidence for the disposition of the dimethylbenzimidazole group in the proposed B_{12} structure. Arndt (18) considered the crystal structure determination of Hodgkin and Pickworth in the B_{12} field "perhaps the most complicated ever carried out." The crystalline hexacarboxylic acid was a key compound in the rapid elucidation of structure. It proved to be uniquely suitable for direct x-ray analysis.

The earlier chemistry of B_{12} was reviewed by Folkers & Wolf (1), Robinson (19), and Smith (20). Petrow & Ellis (21) summarized work leading to the B_{12} structure proposal.

Properties.—Ericson & Nihlén (22) reinvestigated the electrophoretic mobility of B_{12} and found that its isoelectric point is at pH 1.5 not 1.9 as earlier reported (23). The electrophoretic mobility-pH curves of B_{12} and B_{12}-Factor III are identical. Maddock & Pinto Coelho (24) re-examined the problem of the retention of Co^{60} after neutron irradiation of B_{12} because of contradictory claims in the literature. They observed a retention of about 5 per cent in confirmation of Smith's claim (25).

Stability.—Shenoy & Ramasarma (26) found that the iron present in crude liver extracts was an important factor in stabilizing B_{12} against destruction by heat and alkali. B_{12} could be further protected by addition of ferric chloride to the liver fraction. B_{12} was slowly inactivated in mixture with other vitamins including thiamine and niacinamide (27) and more rapidly at elevated temperatures (28). Graham & Hier (29) found that a water-soluble fraction from the digestive organs of cattle and hogs would stabilize B_{12} in the presence of ascorbic acid, thiamine, and niacinamide. The stabilizing factor is thermolabile and is reported not to be related to IF or B_{12} binding.

B_{12}-Factor III.—This is the only B_{12}-like pigment (2, 30) encountered thus far in nature which has B_{12} activity in animals and man (31). It differs from B_{12} in having 5-hydroxybenzimidazole as the nucleotide base. The base was identified independently by Friedrich & Bernhauer (32, 33, 34) and by Robinson *et al.* (35). From an acid hydrolysate the German workers isolated Factor B and a crystalline nucleotide which on further hydrolysis yielded the crystalline base identical with the synthetic compound. The Merck group isolated the base as its picrate and hydrochloride and found

them identical with the synthetic salts. They also obtained evidence for the occurrence of the nucleoside and nucleotide of the base in the Factor III hydrolysate. The positional relationship of the hydroxyl group to the glycosidic bond has not been established.

Occurrence.—In animal tissues B_{12} occurs in conjugated form. Hedbom (36) reported on the further purification and properties of the cobalamin polypeptide fraction isolated from bovine liver by Wijmenga *et al.* (37). By means of electrophoretic column chromatography two cobalamin compounds were separated from a protein fraction. The major cobalamin compound appeared homogeneous with a molecular weight of less than 10,000. Only after treatment with KCN was cobalamin extractable from its aqueous solution. The water-soluble peptide portion remaining was found to contain glycine, alanine, valine, leucine, threonine, aspartic acid, glutamic acid, tyrosine, and histidine.

A number of reports appeared on the B_{12} activity in sewage. Whitemarsh *et al.* (38) studied the B_{12} activity of sewage sludges and confirmed in general the findings of Hoover *et al.* (39) (average microbiological value 10 μg./gm. solids). Limited chromatographic study pointed to the bulk of the activity being attributable to B_{12}. In an extensive analytical study on different types of sludge from a number of sewage plants over a period of three years Neujahr (40, 41) found values of the same general order of magnitude ranging up to 25 μg./gm. solids depending on the nature of the incoming fresh solids and the sewage treatment. The activated sludge process gave higher values than simple digestion with 80 to 90 per cent of the B_{12} activity being attributable to cyanocobalamin and the remainder principally to Factor A, pseudo B_{12}, and Factors C_1 and C_2. In the course of activation the percentage of cyanocobalamin increased from an average of about 50 per cent, the increase occurring at the expense of B_{12}-like pigments including Factor B. In these studies Factor III would have been included in the cyanocobalamin fraction. Bernhauer & Friedrich (2) found a wide variation in the B_{12} activity of sewage sludges from 40 different plants in West Germany with values of 3 to 10 μg./gm. solids being encountered most frequently. Details of their isolation studies on the B_{12} pigments in sewage sludge have appeared (2, 42, 43, 44). Concentrates were prepared by the usual methods, and the pigments were separated by chromatography over alumina and cellulose powder columns. Five pigment fractions were obtained, called Factors I to V; Factors II, III, and IV were crystalline. Factor II was identified as B_{12}, Factor IV as pseudo B_{12}, and Factor III as the 5-hydroxybenzimidazole analogue of B_{12}. Factor I was identified as Factor B while Factor V is apparently a mixture of microbiologically inactive, nucleotide-free acidic pigments perhaps similar to or identical with microbiologically inactive pigments encountered by other workers in feces (3) and in cultures of a rumen microorganism (45). In the course of their preparative work Bernhauer *et al.* (42) made a detailed study of the efficiency of a variety of phenols in extraction and precipitation of the B_{12} pigments. They found that *m*-

and *p*-chlorphenols were most efficient. Small amounts of phenols precipitated the pigments from dilute aqueous solution as insoluble molecular complexes. Polar organic solvents were most efficient in transferring the pigments back to water. Friedrich *et al.* (43, 44) studied the influence of salts on the chromatographic separation of the pigments on cellulose in *sec*-butanol-water mixture. The efficiency of separation was markedly enhanced by the addition of sodium or potassium salts of monobasic polyatomic acids such as perchloric, tetraphenyl-boric, camphorsulfonic, or trichloroacetic. Brown *et al.* (46) isolated a number of new B_{12}-like pigments from pig and calf manure which they refer to as Factors D, E, F, G, H, and I in continuation of the series, Factors A, B, and C of Ford & Porter (47). Factors F, G, H, and I were obtained in crystalline form. The nucleotide bases in Factors G and H are hypoxanthine and 2-methylhypoxanthine, respectively. They appear to differ from Factor A and pseudo-B_{12} only in having oyxgen instead of an amino group on the purine ring. Factor I appeared identical with Bernhauer's Factor III. Both are effective in pernicious anemia. Factors D and E are acidic while F is electrophoretically neutral. Factor D is microbiologically inactive, while E and F are, like Factor B, active on *Escherichia coli* but inactive on *Lactobacillus leichmannii*, *Euglena gracilis* and *Ochromonas malhemensis* (3). The difficulties in this field are exemplified by the fact that Factors G and H behaved identically on chromatography and electrophoresis under a variety of conditions. They were separated through electrophoresis of their dicyano-complexes. The fact that many of the B_{12}-like pigments are available only in minute amounts and some in amorphous form increases the complexity of the problem. New methods and further refinements of existing methods are needed for separation and characterization. For proof of identity great reliance has been placed by all workers on paper chromatography, electrophoresis, and microbiological activity on a limited number of organisms. Peterson *et al.* (48) found that pigments which appeared identical by the usual methods except for small differences in crystallographic indices could be readily distinguished by extending the microbiological spectrum.

Of interest is Klosa's (49) isolation of 41 mg. of red crystals from 500 kg. of cow manure that had been aged. Although the author considered the product to be B_{12} it would be surprising if it did not contain pseudo-pigments. Janicki & Pawelkiewicz (50, 51) found that *Propionibacterium shermanii* produced a small amount of B_{12} and a large quantity of an amorphous pigment referred to as B_{12p}. B_{12p} has properties very similar to those of Factor B. They may be identical.

Biosynthesis of B_{12} and B_{12}-like compounds.—Fantes & O'Callaghan (17) prepared the crystalline benzimidazole analogue of B_{12} by feeding *o*-phenylenediamine to *Streptomyces griseus*. *o*-Phenylenediamine and 1,2-diamino-4,5-dimethylbenzene were found to compete for the nucleotide base linkage. It has been well established by Ford & Holdsworth (52) and Friedrich & Bernhauer (2) that *E. coli* can synthesize a complete B_{12} pigment from

Factor B and a nucleotide, nucleoside, or base. Judging from the yields it appears that the organism first degrades the nucleotide or nucleoside to the base before incorporation into the finished molecule (53). Ford *et al.* (16) offered evidence for the directed synthesis of B_{12}, pseudo B_{12}, or Factor A on Feeding Factor B and the corresponding nucleotide base to *E. coli* 113-3. B_{12} and Factor A were also formed on feeding the corresponding nucleotide. However, feeding adenosine or adenylic acid did not result in the formation of pseudo B_{12}, pointing to the possibility that the adenylic acid in pesudo B_{12} may have an α-glycosidic linkage. These observations are in keeping with the view (Hodgkin) that the molecular structure of B_{12} will accommodate only purine 7-α-sugar derivatives (54). In this connection it is of interest that Kaplan *et al.* (55) recently isolated a DPN isomer containing nicotinamide in α-linkage. However, as Dellweg *et al.* (53) point out, a somewhat similar situation exists in the rat in which Roll & Weliky (56) find that adenine is incorporated more efficiently than either adenosine or adenylic acid into the RNA of the viscera. The weight of available evidence favors the view that the biosynthesis of a "complete" cobalamin is effected by some other route than the direct coupling of a nucleotide with Factor B.

Adenine and 2-methyladenine are the only natural purines that *E. coli* can build into a B_{12}-like pigment, while no pyrimidines are utilized in this way (16, 57). A number of adenine derivatives such as 2,6-diamino purine, 2,8-dichloro-adenine, and 2-methylthio-adenine, served as precursors of new unidentified factors as did benzimidazoles substituted in the 5 or 6 positions or both with nitro, amino, or chloro groups (paper chromatographic and ionophoretic evidence) (16). Dellweg *et al.* (53) used a wild strain of *E. coli* to synthesize a variety of B_{12}-like compounds from *o*-phenylenediaamines and benzimidazoles. In the case of 5,6-dimethyl-, 5-methyl-, 5,6-diethyl-, 5,6-dichorobenzimidazole, and benzimidazole itself, the corresponding cobalamins were isolated in cystalline form, characterized, and proved to have the respective nucleotide bases by degradation. All these compounds were highly active for the growth of *E. coli, L. leichmannii* and *O. malhemensis*. It would appear that the 2,4 or 7 positions or both of benzimidazole must be free or potentially free in order that the compound be incorporated into an analogue (53, 58, 59, 60). Friedrich & Bernhauer (57) noted that those benzimidazoles which form molecular compounds with 2,4-dinitrophenylhydrazine also serve as presursors for the biosynthesis of cobalamins. Pawelkiewicz (58, 59, 60) used *P. shermanii* to synthesize B_{12} and a number of "unnatural" cobalamins including the 5(6) methylbenzimidazole analogue. The latter product prepared in crystalline form had physical and microbiological properties very similar to those of B_{12}. Some evidence was obtained that benztriazoles and benzthiazoles served as precursors (16, 58). Robinson *et al.* (35) synthesized Factor III with *E. coli* from Factor B and 5-hydroxybenzimidazole. A second new crystalline cobalamin was also isolated, probably the benzimidazole position isomer of Factor III. No

B_{12}-like compound has yet been obtained which has anti-vitamin activity.

Assay.—A discussion of methods for the measurement of B_{12} (61) was part of a symposium on the biochemistry of this vitamin. Another conference (61a) covered a comparison of various microbiological assay methods and the results obtained by them with biological assays using rats and chicks. Wolff *et al.* (62) claimed good results in assaying B_{12} in human serum with *L. leichmannii* when HCN was added to the assay medium before autoclaving. Although neither this organism nor *E. coli* has been considered too useful for assaying B_{12} in serum, a modified *E. coli* mutant assay was reported for this purpose (63). The addition of methionine-free casein hydrolysate to a mineral medium allowed better growth of the assay organism, and results were obtained in 40 to 48 hr. compared to 7 to 9 days for *E. gracilis*. Hine and co-workers (64) reported an inhibitory effect of copper on the *E. coli* mutant when used in the tube assay. Copper was less than one twentieth as inhibitory when the organism was growing on methionine as when growing on B_{12}, suggesting that copper exerted its effect at some stage of the synthesis of methionine in which B_{12} is concerned. McLaughlan *et al.* (65) observed that assaying the B_{12} content of pharmaceutical products by the *L. leichmannii* method gave lower results than the tube assay with *E. coli* mutant 113-3. This was shown to be a result of the thiamine present, the effects of which could be eliminated by treatment of the samples with sodium metabisulfite. Yragui *et al.* (66) developed a synthetic assay medium for *L. leichmannii* which contained amino acids in place of the customary hydrolyzed casein. Serial propagation of stock cultures in the complete synthetic medium resulted in adapted populations which were B_{12}-independent. Substituting soybean protein hydrolysates for amino acids prevented adaptation. Williams *et al.* (67) presented modifications and improvements of the *Ochromonas* and *E. coli* methods as well as details of a chick growth method for assaying B_{12}. It was concluded that pseudo B_{12} does not occur commonly in B_{12} feed supplements and that the USP *leichmanni* assay may be safely used for products of this nature although occasionally a check assay should be made with *Ochromonas* and chicks. Certain fermentation materials contain substances that inhibit chick growth, so not even this assay can be depended on in all cases.

Although previous B_{12} assay procedures in rats have employed thyroid-treated animals, Sherman *et al.* (68), using weanling rats from breeder females that had received a vegetable protein ration during gestation and lactation, obtained a graded response to B_{12} but no response to pseudo B_{12}. However, the addition of unidentified factors from liver, etc., gave a further response, indicating that the test procedure was not specific for B_{12}.

A modification of the chemical assay method based on liberation of HCN by light exposure was reported by Tuzson & Vastagh (69). Fortune (70) reported the routine use of this general method in the control of pharmaceutical products and claimed an accuracy of ± 5 per cent. Mader & Johl (71) developed a scheme for routine analyses of fermentation and com-

mercial B_{12} concentrates which consisted of countercurrent separation followed by either spectrophotometric or microbiological assay. This method was claimed to differentiate B_{12} from the pseudo B_{12} group.

Vitamin B_{12} Function

Extensive reviews appeared which discussed the function of B_{12}. A review by Heinrich & Lahann (71a) covered the physiology, pathology, and biochemical mechanism of B_{12}; one by Ungley (72) discussed the chemotherapeutic action of this vitamin. The latter author stressed the point that in spite of the many papers that have mentioned the empirical use of B_{12} in treating various disease conditions, the only rational and proven successful therapeutic use is in the megaloblastic anemias. Ford & Hutner (3) provided a stimulating discussion of the role of B_{12} in the metabolism of microorganisms.

Bacteria.—The function of B_{12} in bacterial nutrition was reviewed by Stokstad *et al.* (73). The metabolic interrelationships of B_{12} and FA were discussed by Williams (74).

The work of Lascelles & Cross (75) led them to conclude that microorganisms requiring B_{12} fall into two groups: those whose B_{12} requirement is satisfied by methionine but which do not respond to deoxyribosides, and those for which only deoxyribosides substitute for the vitamin and which require methionine even in the presence of B_{12}. Saxena *et al.* (76) reported that the addition of B_{12} to a growing *E. coli* culture inhibits the synthesis of thiamine. Confirming previous work in which he found that B_{12} added to cultures of *E. coli* reduced the free pantothenic acid excreted into the medium, Jännes (77) also showed that the presence of B_{12} in *E. coli* medium had no effect on the coenzyme A content of the cells. It was reported by Ottey & Daniel (78) that the inhibition of acetate oxidation, by fluoroacetate in old cells of *E. coli* 113-3 adapted to acetate metabolism, was reversed by B_{12}. Lockhead & Burton (79) continued their work on the relation of B_{12} to the metabolism of soil organisms, describing the isolation of 10 types of bacteria for which B_{12} is an essential nutrilite. Neither thymidine nor methionine effectively substituted for B_{12} in these organisms. Using a simplified medium, Johnson *et al.* (80) investigated methyl synthesis in *O. malhamensis*, the organism noted for its specific growth response to B_{12}. In this medium methionine but not homocystine gave a response in the absence of B_{12}. In the presence of B_{12}, homocystine plus glycine or serine replaced methionine. These authors explained the failure of Miller & Yeager (81) to duplicate these results as possibly attributable to a shorter incubation period used by the latter workers. Soldo (82) presented evidence for a role of B_{12} in nucleic acid synthesis by showing that B_{12}-deficient *Euglena* were 12 per cent lower than normal cells in total nucleic acid although there was no alteration in RNA to DNA ratio. Peterson *et al.* (48) reported comparative assay values, obtained with a variety of microorganisms, for a group of pseudo B_{12}-type compounds containing different nucleotides and

their deaminated analogues, as well as factor B which contains no nucleotide. These differential assays provided evidence for distinguishing between these compounds which are chemically differentiated with difficulty in some cases. All were inactive for *Ochromonas*. It was observed by Ford *et al.* (83) that *O. malhamensis* growing with a minimum of B_{12} was apparently competitively inhibited by the addition of rather large amounts of pseudo B_{12}. These workers believed this phenomenon was a result of the presence of a substance in *O. malhamensis* or its filtrate which combines with B_{12} or pseudo B_{12} and renders them undializable.

Animals.—Bruemmer *et al.* (84) reported that a B_{12} deficiency in rats interfered with the brain development of the offspring, which usually died within 2 to 3 days after developing a high blood urea, low liver glycogen, and a deranged ratio of DNA to RNA in the brain tissue. It was shown by Jones *et al.* (85) that female rats deprived of B_{12} from before mating until the end of gestation produced deficient and weak progeny that were below normal in body weight and showed pathological changes in many tissues, especially the liver, heart, and kidneys. Fatterpaker *et al.* (86) found that the tissues from hyperthyroid rats receiving no B_{12} showed a reduced acetylating ability compared to tissues from hyperthyroid rats receiving B_{12}. They concluded that thyrotoxicosis is primarily a deficiency of B_{12} with a consequent interference in the generation and utilization of energy-rich bonds coupled with electron transport. This interesting observation needs confirmation. Thyroid has commonly been fed to rats to produce a B_{12} deficiency, but it was considered to be a general stress phenomenon whereas this work implies that there is a specific relationship between B_{12} and thyroid activity.

In a study of fasting chicks Charkey *et al.* (87) observed a rise in the free amino acid level of the blood which was moderated by B_{12} at levels in the diet above that required for maximal growth. It was found by Kline (88) that B_{12}, as has been reported for FA, is essential for optimum response of the chick oviduct to stilbestrol. This action is not related to PGA in the sense of promoting its conversion to metabolically active forms. Antibiotics are known to spare B_{12}, as well as other vitamins. Jenkins *et al.* (89) helped explain this by showing that antibiotics may exert an effect on the rate and site of B_{12} synthesis, as well as on its utilization. Synthesis of B_{12} was observed as far forward as the proventriculus in the digestive tract of the chick. Jackson (90) showed that B_{12} normally present in the ceca of the hen is essentially unabsorbed. In attempting to explain the growth depression caused by high dietary lard on B_{12}-deficient chicks Fox *et al.* (91) found it could not be attributed to depletion of B_{12} in the liver. Briggs & Fox (92) assayed des-dimethyl B_{12} on chicks using this assay diet and found it to be only 9 per cent as active as B_{12} although it has been reported (17) to be fully as active as B_{12} clinically. The results of experiments by Arscott *et al.* (93) indicate that 1,2-dichloro-4,5-diaminobenzene (which is an analogue of an apparent precursor, 1,2-dimethyl-4,5-diaminobenzene, of B_{12}) and

2,5-dimethylbenzimidazole inhibit the hatchability of eggs, probably by interfering with B_{12} metabolism, although it was not found whether B_{12} would reverse the inhibition. Evans *et al.* (94) assayed the B_{12} content of fresh eggs and obtained a value of 387 mμg. B_{12} per egg, only 25 mμg. of which was in the white. The egg of the bobwhite was found by Dale (95) to contain appreciable B_{12} although there was none being consumed in the diet. Thus this species can apparently utilize the B_{12} formed in its own digestive tract.

Hopper & Johnson (96) injected pseudo B_{12} into B_{12}-deficient calves maintained on a synthetic diet. As judged by weight gain pseudo B_{12} was inactive, but the liver of one calf (out of two examined) contained B_{12} activity as indicated by *Ochromonas* assay.

A review of B_{12} and cobalt in the nutrition of ruminants was published by Kon & Porter (97). B_{12} activity in rumen contents and excreta of sheep as determined by four microbiological assays was reported by Hine & Dawbarn (98, 99). The *E. coli* plate assay gave results about 20 times higher than the *Ochromonas* assay. Differential assays indicated two or more B_{12}-like pigments to be present in urine. In contrast to the pseudo B_{12}-like nature of the pigments in sheep feces reported above and other published data for calf feces (97), Moinuddin and Bentley (100) reported that extracts of steer feces assayed as high on chicks and rats as on *L. leichmannii*. Kon (101) has emphasized the apparent selective mechanisms of absorption of the B_{12}-like pigments in the ruminant.

The B_{12} requirement of monkeys was found by Wilson & Pitney (102) to be more than 2 μg. and less than 10 μg. per day. Woolley (103) attempted to confirm his previous finding that mammary cancers of mice synthesized B_{12}. The results were equivocal, and some strains of tumors appeared to destroy B_{12}. Confirming previous reports of the low toxicity of B_{12}, Levin (104) was unable to establish an LD_{50} in mice. What would be equivalent to a 150-gram dose in a human produced no effect in mice, other than red eyeballs. Rigdon *et al.* (105) found that B_{12} influences the process of hepatic repair following injury resulting from selenium intoxication. Ferguson *et al.* (106) made a histological study of 17-day embryos from hens fed B_{12}-deficient diets with and without added B_{12}. B_{12} deficiency caused the following changes in the embryos, which did not occur when B_{12} was present: decrease in size; edema and hemorrhages; necrosis in liver, brain, and spinal cord; and a marked increase in fat in the parenchymatous tissues.

Metabolism of methyl groups.—Most workers agreed that B_{12} is required by animals for methyl synthesis but not for transmethylation reactions. Thus Chang & Johnson (107) reported that adding B_{12} to the diet of baby pigs caused a fivefold increase in incorporation of the α-carbon of glycine into the methyl group of choline but no effect on the incorporation of the α-carbon of glycine into serine or ethanolamine. Mistry *et al.* from the same laboratory (108) showed that formate was active in preventing fatty livers in baby pigs and that B_{12} did not affect the use of this donor for choline synthesis. One

per cent serine was as effective as choline in protecting against fatty livers. In still another study at the Illinois laboratory (109) it was shown in baby pigs that glycine would serve as a source of methyl groups for choline synthesis to prevent fatty livers only in the presence of B_{12}. Verly & Cathey (110) found that rats deficient in B_{12} incorporated less C^{14} from radioactive methanol into the methyl group of choline than controls. Young & Lucas (111) showed that B_{12} caused a growth response in rats receiving diets with insufficient betaine to methylate the monoethanolamine present. This methyl synthesis caused by B_{12} was able to replace about half of the necessary betaine in the diet. Arnstein (112) pointed out that while B_{12} is required by rats and chicks for labile methyl group synthesis but not for transmethylation reactions, a more important function may be catalyzing the formation of the methyl group of thymine. Stekol concluded in a summary of work in his laboratory (113) that B_{12} is not involved in any transmethylation reaction in rats, mice, or chicks but that it is involved in the utilization of the alpha carbon of glycine for the synthesis of the methyl group of methionine and one methyl group of choline.

On the basis of experiments with liver homogenates from pig, chick, and rat Mistry *et al.* (114) concluded that B_{12} is not involved in direct transmethylations from betaine to homocysteine or in the reduction of homocystine to homocysteine. On the other hand, Ericson *et al.* (115) reported that a B_{12} deficiency decreased both the activity of the betaine-homocysteine transmethylase of rat liver and the ability of the liver to synthesize methyl groups. They concluded (116) that this decrease in the betaine-homocysteine transmethylase activity of the liver was specifically attributable to a B_{12} deficiency and not to a deficiency of any other vitamin.

Other effects of B_{12} possibly related to methyl group transformations were reported. Bennett *et al.* (117) noted certain morphologic changes in the livers of rats fed a labile methyl-free diet which resembled the preneoplastic changes produced by *p*-dimethylaminoazobenzene. These changes were accelerated by a B_{12} deficiency. In trying to account for his lack of success in producing renal lesions in young male rats on a low choline diet, Mulford (118) found that if the mothers of such rats received as little as 0.06 μg. B_{12} per gram of food during the period from birth to weaning of the young the offspring had a very low incidence of hemorrhagic kidneys. Shils *et al.* (119) observed a type of fatty liver in rats in which the lipide was localized in the portal area. This occurred on diets in which the protein was derived from corn meal, casava, or rice, and the condition did not respond to the addition of choline, B_{12}, or methionine to the diet which suggests that something other than methyl group deficiency was responsible. The effect of B_{12} and FA on the formation of lipides was observed by Grunbaum *et al.* (120) in an entirely different setting. They observed that these two vitamins prevented the *in vitro* formation of the fat globules often noted in cultures of chick heart fibroblasts, especially older cultures.

Absorption and metabolism in man.—Schwartz and co-workers (121) ob-

served that parenteral B_{12} in small doses provided an adequate treatment of pernicious anemia, but the daily oral administration of 25 μg. B_{12} with 1.67 mg. PGA proved inadequate for the maintenance of the patient. Reisner *et al.* (122) and also Conley & Krevans (123) maintained pernicious anemia patients for long periods with 1 mg. weekly oral doses of B_{12}. In the former study the serum B_{12} levels were apparently adequate, but in the latter case they were subnormal, indicating that possibly this dosage was suboptimal.

Mollin & Baker (124) pointed out the advantage of using the short-lived isotope of cobalt (Co^{58}) in studying the absorption of physiological amounts of B_{12} since repeated studies on the same individual can be made. Chow & Okuda (125) studied the variables affecting the Schilling test for measuring the absorption of orally administered B_{12}. They found that only a minor portion of the administered B_{12} appeared in the urine regardless of the flushing dose or frequency. Pretreatment of the subjects with massive doses of B_{12} can distort the results.

B_{12} seems to be absorbed through the nasal mucosa without the aid of IF. Monto *et al.* (126) obtained complete response in two patients with 150 to 200 μg. doses and suggested (127) that B_{12} is absorbed directly by the nasal mucosa with a urinary excretion pattern similar to that obtained after parenteral injection. Later (128) these authors reported on results with a large number of patients who were successfully treated by the intranasal route. Unglaub & Goldsmith (129) found that 3000 μg. oral doses of B_{12} at four-week intervals without IF produced satisfactory hematological remissions in pernicious anemia and nutritional macrocytic anemia. More evidence that small changes in the B_{12} molecule greatly affect absorption was furnished by Rosenblum *et al.* (130) who found that normal humans absorbed chlorocobalamin much less effectively than cyanocobalamin even though these compounds have identical activity when injected into pernicious anemia patients.

Whitby (131) reviewed the history, biochemistry, and treatment of pernicious anemia. Mueller & Will (132) described the relationship of B_{12}, FA, and ascorbic acid in the treatment of megaloblastic anemias. It was observed by Thompson & Ungley (133) that megaloblastic anemia which had developed because of intestinal strictures resulting from operations did not respond well to B_{12} therapy.

Since it is known that plants are essentially devoid of B_{12}, it is interesting to contemplate the B_{12} status of vegetarians. Wokes *et al.* (134) have investigated a group of strict vegetarians, called vegans in Great Britain, and found that a high percentage of them exhibited B_{12} deficiency as indicated by serum B_{12} levels and neurological symptoms. The serum B_{12} levels among this group were the only clear indication of B_{12} deficiency, ranging from 45 to 193 μμg. per ml. compared to 200 to 320 for normals. There was little anemia probably because the diet was high in FA. It was suggested by Jewesbury (135) that since neurological changes sometimes occur in patients with normal peripheral blood counts, the general term B_{12} deficiency should

be used and this qualified by adding megaloblastic anemia or neuropathy as the case warrants. In an interesting finding requiring confirmation, Wild *et al.* (136) noted changes in amino acid concentrations in the blood after injection of B_{12}, indicating a stimulation of protein synthesis. They claimed this effect was shown even in subjects who had an adequate supply of the vitamin. Speculating on the role of B_{12} in human nutrition, Wokes & Picard (137) believe that it competes in the liver with transulfurase for CN. Liver CN which is not taken up by hydroxocobalamin will be converted to thiocyanate by transulfurase and an S-donor and excreted in the urine. As evidence for this it was claimed that people with B_{12} deficiency excrete increased amounts of thiocyanate in the urine. This can be overcome by giving large doses of B_{12}.

Several studies were reported of B_{12} determinations in body tissues using *E. gracilis*, an organism well suited for this purpose because of its extreme sensitivity although it grows very slowly and has been shown to respond to pseudo B_{12}. Pitney *et al.* (138) found that normal human livers contained an average of 0.28 μg. B_{12} per gram wet tissue and that it existed there as a loosely bound protein complex which could be utilized by *E. gracilis* as readily as the free vitamin and did not require preliminary heating procedures necessary in the analysis of serum. Boger *et al.* do not agree than *E. gracilis* is the best organism for assaying B_{12} in serum. They pointed out (139) that assays by methods requiring clarification of the serum, as is necessary with *E. gracilis* because growth is measured by turbidity readings, result in low values since removing the protein after heating also removes some of the B_{12}. These workers prefer *L. leichmannii* with the response being determined by titration after 72 hr. In this procedure the serum is simply diluted out so that the coagulated protein remains in contact with the growing bacteria which utilize all the B_{12} present. They obtained an average value of 560 $\mu\mu$g. B_{12} per ml. for 528 normal persons, ranging from 200 to 1000 $\mu\mu$g. per ml. There was a trend toward lower serum concentrations of B_{12} with advancing age. Lear *et al.* (140), using *E. gracilis*, reported an average serum B_{12} level of 532 $\mu\mu$g. per ml. in 20 normal individuals. All had free B_{12} present. In 33 patients with pernicious anemia in relapse the average value was 39 $\mu\mu$g. per ml., and no free B_{12} was detected.

Tasker (141) found, in cases with megaloblastic anemia, a close correlation between the serum B_{12} level and the urinary excretion of FA after a 5 mg. parenteral loading dose of the latter, the higher the serum B_{12} the higher the excretion of FA. This supports a previous suggestion that the excretion of FA, following a loading dose, does not measure the tissue depletion but indicates the degree of B_{12} deficiency. Harris (142) observed an aggravation of clinical manifestations of FA deficiency which developed in patients with megaloblastic anemia during therapy with 1 μg. daily parenterol doses of B_{12}; FA promptly reversed these deficiency manifestations.

Beard *et al.* (143) observed an interesting correlation between B_{12} serum levels and certain forms of leukemia; those with acute lymphocytic leukemia

had normal serum B_{12}, but those with myelocytic leukemia showed greatly elevated serum B_{12}. A correlation between serum B_{12} levels, as assayed with *E. gracilis*, and certain liver diseases was observed by Jones & Mills (144). Patients with cirrhosis had serum B_{12} levels 3 to 8 times normal, and those in hepatic coma had values 30 to 40 times normal. These patients also excreted 10 to 30 times the normal amount of B_{12} in the urine. Brebner & Wilkinson (145) found that cells from the marrows of pernicious anemia patients and hyperactive normoblastic marrows contain approximately the same amount of DNA. This suggests that many of the cells of the pernicious anemia marrow are of a proliferative type and are building up DNA in preparation for mitosis, which is blocked for some other reason. Swan *et al.* (146) extended the British work of several years ago on the effect of various metabolites on the growth of marrow cells *in vitro*. They found that B_{12} stimulates conversion of the megaloblast to the normoblast, while culturing in pernicious anemia serum reversed this trend. Folinic acid also stimulated this conversion, but no enhancing effect of intrinsic factor upon the activity of B_{12} in the conversion could be demonstrated.

Patrick (147) studied a group of Jamaican children who received dietary B_{12} supplementation over a period of nine months. No difference was noted in the B_{12} excretion rate or plasma levels of children after a test dose of B_{12} whether (*a*) their growth had been retarded, (*b*) the growth-retarded children had been treated with B_{12}, or (*c*) their growth rate was normal. Grump & Tully (148) gave 25 μg. B_{12} plus 10 mg. B_1 daily to 50 children with a clinical diagnosis of malnutrition and anorexia. There was improved appetite with increased weight and height increments in those of this group who were indicated by Wetzel grid charts to be suffering from simple growth failure. Jolliffe (149) summarized the results of study in which 350 underweight children in two schools in Italy were used. Two seven-month periods of treatment, during which 20 μg. B_{12} or a placebo was given to each child per day, were separated by a five month period without treatment. Significant differences in weight gains were noted between the children receiving B_{12} and their controls, but this advantage was lost during the five month period when B_{12} was not given. However, Campbell & McLaughlan (150) concluded from a study of nine reports on the oral use of B_{12} in normal, underweight, and chronically ill children that there is need for critically controlled experiments before definite conclusions can be reached regarding effects of B_{12} on the growth of children.

Intrinsic Factor

Preparation and properties.—A number of workers reported the preparation of highly active IF concentrates, some claiming apparent purity. However, there was little agreement concerning the characteristics of these preparations. Andresen (151) prepared a B_{12}-protein complex with intense red color, high molecular weight, and a B_{12} content of 14.7 μg./mg. Starting with a commercial preparation of IF from hog stomach mucosa, the final product

had its B_{12} binding activity, measured on *E. coli*, increased 17 times and the amount required per day for complete hematological response in patients with pernicious anemia decreased from 20 mg. to 1.2 mg. Williams and co-workers (152, 153) reported the preparation of an IF concentrate from hog stomach which was active in pernicious anemia patients in relapse in a daily dose of 1 to 2 mg. when given with B_{12}. It contained 11.8 per cent N. This was then separated by ultracentrifugation into high and low molecular weight fractions with unstated activity. Wijmenga & Van Baal (154) obtained IF concentrates from hog pyloric mucosa which were highly active clinically by precipitation with different concentrations of ammonium sulfate. The most active material was precipitated at between 75 and 95 per cent saturation. They claimed there was no correlation between IF activity and blood group substance A or H activity. Heinrich reported (155) that a highly purified B_{12}-protein concentrate prepared by Wijmenga (156) from hog gastric mucosa was clinically active in a daily dose of 0.75 mg. which contained 2 μg. B_{12}. A clinically active B_{12}-protein complex, homogeneous by both ultracentrifuge and electrophoresis tests, was isolated by Andresen & Skouby (157). It contained 18 μg. B_{12}/mg. This preparation was studied further by Holdsworth & Ottesen (158). They reported a nitrogen content of 10.3 per cent and a reducing sugar content of 28 per cent. Several proteolytic enzymes, including papain and trypsin, failed to digest it. Spectrophotometric assay indicated 19 μg. B_{12} per mg., corresponding to a molecular weight of 79,000, assuming a 1:1 combination of protein and B_{12}.

Latner and co-workers (159, 160, 161) have reported on the preparation and properties of an IF concentrate which they believed was the most potent product so far described. It is mucoprotein in nature, has a molecular weight between 10,000 and 20,000, and is homogeneous by the usual tests such as ultracentrifuge, paper electrophoresis, and chromatography. It has blood group substance activity and is a growth factor for *Lactobacillus bifidus*. The clinical tests of this material (162), which were carried out on pernicious anemia patients in relapse, indicated that the effective daily dose was between 1 and 4 mg. per day, given with 5 μg. B_{12}. Although the IF preparations of Van Baal *et al.* (163) contained blood group A and H substance activity, they had no B substance activity. There was no correlation between IF activity and blood group substance activity, but these workers do not exclude the possibility that pure IF might have blood group activity.

New observations were made on the thermostability of IF (164). The IF content of human gastric juice or an IF concentrate prepared from hog stomach lost their activity when boiled at pH 1.2 to 1.4. However, the latter was stable when boiled at pH 6.0. Therefore, the heat stability of IF, which has been reported lately, apparently is not a result of its prior combination with B_{12} since it can also be demonstrated for concentrates boiled in water prior to adding the vitamin.

Assay.—Several groups suggested modifications and improvements of the Schilling technic (165) for assaying IF activity. Toporek *et al.* (166, 167)

and Ellenbogen *et al.* (168) evaluated the limitations of the method under their own conditions and outlined changes which they believe give improved results. For dealing with very low excreted levels of $B_{12}Co^{60}$ Klayman (169) introduced a modification consisting of concentrating the 24-hr. sample of urine prior to measuring the radioactivity. Glass *et al.* (170) tested 11 different commercial IF preparations both by their hepatic uptake method and by the classical hematological method. There was complete agreement. Heinrich (155) described a very specific and sensitive test procedure for IF consisting of a combination of the hematological procedure with a determination of bound and free serum B_{12} and urinary excretion of B_{12} by microbiological assays using *E. gracilis* and *O. malhamensis*. By this procedure he found a higher excretion of B_{12} when purified rather than crude IF was given, indicating a possible inhibitory effect of crude preparations, an observation others have made. A microbiological assay for IF using *E. coli* strain 113-3 was reported by Tixier & Neudörffer (171). It depends on the well-known binding of B_{12} by *E. coli* and the effect of IF on this binding. No comparative clinical data were presented, and this procedure seems open to question.

B_{12} binding.—Some interesting observations on the relationship between B_{12} binding and clinical activity of IF concentrates were made. Unfortunately no standardized means of measuring B_{12} binding has been adopted. As a result it is difficult to evaluate many of the reported data.

Considerable evidence was presented on the nature of binding substances. Gregory & Holdsworth (172, 173) isolated a pure B_{12}-protein complex from sow's milk containing 23.6 μg. bound B_{12} per mg. It was glycoprotein in nature, was calculated to have a molecular weight of 55,000, and contained 16 per cent N. From comparative B_{12} binding experiments with the milk of several other species and with binding fractions from hog stomach mucosa, it was concluded that a specific protein common to all these sources was responsible for the binding. Studies were made on the nature of the linkage of this protein with B_{12}, but no conclusion was reached. Cresseri (174) reported on the properties of a highly purified B_{12} binding preparation. Its B_{12} binding capacity was 22.1 μg. B_{12} per mg. However, it had only 8.2 per cent N compared to the 16 per cent found above (173).

In a study of absorption of B_{12} in chicks and rats it was found (175) that binding substance from pig stomach made B_{12} combined with it less available than the free vitamin. Coates *et al.* (176) studied the availability of bound B_{12} from various sources in the diets of chicks and rats. All B_{12}-protein complexes were fully active parenterally, but their oral activity varied greatly according to the source. However, there was no strict species specificity. These results agree with those of Rosenblum *et al.* (177) and Clayton *et al.* (178) who found that the presence of excessive IF inhibited the absorption of B_{12} in normal and gastrectomized rats.

It was shown by Raine (179) that highly purified IF bound B_{12}, but the amount bound was proportional to the concentration of B_{12} added This effect was likened to clinical observations that increasing either B_{12} or IF

increases hematopoietic effect in oral therapy of pernicious anemia. Bishop *et al.* (180) studied the relationship of binding power to IF activity in patients with total gastrectomy. This avoided the complication introduced by the binding power of the gastric juice from patients with pernicious anemia. They found that $B_{12}Co^{60}$ bound by IF was preferentially absorbed over free B_{12} given at the same time and vice versa. They concluded that binding power is necessary for IF activity. Jännes (181) found that, contrary to earlier observations, cells of *E. coli* killed by sonic waves are capable of absorbing B_{12} from the medium.

Mode of action.—The status of the relation of IF to B_{12} in therapy was reviewed by Ungley (72). Schilling (182) discussed what is known about the effect of IF on B_{12} absorption in normal humans, including evidence that the stomach is the only source of IF. Evidence for this latter observation was also furnished by Paulson & Harvey (183). Pitney & Beard (184) also agreed that the stomach is the only source of IF and pointed out that B_{12} serum levels are maintained even at the expense of depleted body stores. This was not in agreement with Conley & Krevans (123), who successfully treated pernicious anemia cases with doses of B_{12} so low that serum levels never reached normal values. Blackburn *et al.* (185) reported that oral treatment with B_{12} plus IF was not as reliable as parenteral therapy in new cases of pernicious anemia or in cases previously maintained by injection of B_{12}.

Glass *et al.* (186) observed a different metabolic pattern for injected B_{12} compared to oral B_{12} taken with IF. Taken orally B_{12} produced a reticulocyte response without a rise in serum B_{12} or any loss in the urine. Injected B_{12} caused a transient rise in serum B_{12} followed by a 60 per cent loss in the urine. These same workers (187) observed an optimum ratio between IF and B_{12} for best results. When the amount of IF exceeded this ratio B_{12} absorption declined.

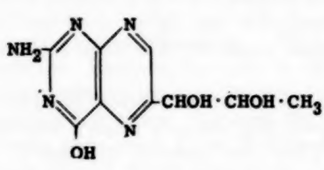
Celli (188) studied the return to normal of gastric mucosa and bone marrow following treatment of pernicious anemia patients with oral B_{12} and IF; these two processes paralleled each other. It was shown by Schilling *et al.* (189) and MacLean (190) that gastrectomy patients soon develop a B_{12} deficiency unless IF is given. They act essentially as pernicious anemia patients. These authors pointed out the usefulness of the Schilling test for detecting potential pernicious anemia patients not yet showing clinical symptoms because of their intake of FA or B_{12} plus IF from vitamin preparations.

Chow *et al.* (191) confirmed an earlier observation by Rosenblum *et al.* (177) that IF from hog stomach mucosa inhibits the absorption of B_{12} by normal rats. They also reported that gastrectomized rats failed to absorb $B_{12}Co^{60}$ when given with IF. This does not entirely agree with the work of Clayton *et al.* (178), who fed 16 mμg. doses of B_{12} to normal rats with 43.5 per cent being absorbed. In gastrectomized rats only 7.1 per cent of the same dose was absorbed, and giving IF with this dose of B_{12} to gas-

trectomized rats increased absorption to 16.8 per cent but could not return it to normal. Watson & Florey (192) fed normal rats 5 mμg. $B_{12}Co^{60}$ and found 66.5 per cent of the dose in the feces. Gastrectomized rats given the same dose excreted 93.8 per cent, and this was decreased to 69.5 per cent when an extract of rat stomach was given with the B_{12}. A fraction from pig pyloric juice did not consistently reduce the B_{12} excretion. Other results indicated partial species specificity. No real progress was made in our understanding of the basic mechanism whereby IF facilitates the transfer of B_{12} across the gut wall.

The Folic Acids and Related Natural Pteridines

Crithidia factor.—In a study of the nutritional requirements of the protozoan *Crithidia fasciculata*, a trypanosome parasite of the mosquito, Cowperthwaite *et al.* (193) succeeded in developing a synthetic medium which required the addition of large amounts of PGA. The PGA could be replaced by liver extract and other natural materials. Pteroic acid was also active as was CF in the presence of adenosine. However, the known breakdown products of PGA or its derivatives failed to account for the activity of liver extract. Conditions of culture were further defined by Nathan & Cowperthwaite (194, 195). Using human urine as a source Patterson *et al.* (196) isolated a new pteridine, $C_9H_{11}N_5O_3$, which satisfied the growth requirements. Oxidation with periodate in acid solution yielded 2-amino-4-hydroxy-6-formylpteridine and in alkaline solution the corresponding 6-carboxy compound. From these degradation results and other ancillary data they assigned the structure 2-amino-4-hydroxy-6(1′,2′-dihydroxypropyl) pteridine and suggested the name biopterin (VI). This same compound was isolated independently by Forrest & Mitchell (197) from wild type Drosophila in their work on the nature of the red eye pigments. Periodate oxidation yielded 2-amino-4-hydroxy-6-formylpteridine and acetaldehyde. The isolated compound compared favorably with a synthetic product obtained by the condensation of rhamnotetrose and 2,4,5-triamino-6-hydroxypyrimidine in the presence of hydrazine. The synthetic product was a mixture of the 6 and 7 isomers. Patterson *et al.* (198) compared the compound from urine with 2-amino-4-hydroxy-6-[1′,2′-dihydroxypropyl-(L-erythro)]-pteridine iso-



VI
Biopterin

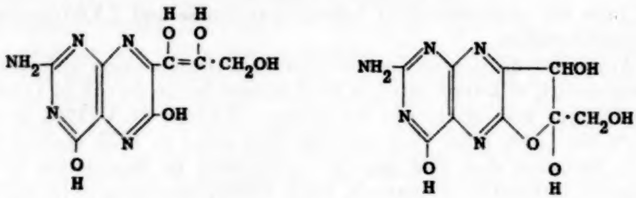
lated from the condensation of 5-deoxy-L-arabinose and 2,5,6-triamino-4-hydroxypyrimidine.

2-Amino-4-hydroxy-pteridines substituted in the 6-position with methyl, hydroxymethyl, or formyl acted as PGA sparers in the growth of *Crithidia fasciculata* but were inactive in the absence of added PGA (196). Several pteroylamino acids other than glutamic also acted as PGA sparers. The authors speculate that this may be attributable to degradation to the pteridine-6-methylol or to biopterin, itself. During the course of its use as a test organism (196) *C. fasciculata*, however, lost its requirement for added PGA. Biopterin alone then stimulated growth while the PGA sparers mentioned above were inactive. That PGA is involved in the metabolism of this protozoan was shown by the reversal of inhibition of growth with 2,4-diamino-5-*p*-chlorophenoxy-6-ethyl-pyrimidine by PGA. Broquist & Albrecht (199) estimate the concentration of biopterin in normal human urine as 1 mg. per liter, which is about the same as that found for xanthopterin by Koschara (200).

Other natural pteridines of possible related metabolic interest.—The growth activity of a simple pteridine like biopterin focuses attention on the companion pteridines in *Drosophila* and other natural materials. Forrest & Mitchell (197, 201, 202) isolated four other pteridines from *Drosophila:* N^8-lactyl-7,8-dihydro-2-amino-4-hydroxypteridine-6-carboxylic acid, isoxanthopterin, 2-amino-4-hydroxypteridine, and 2-amino-4-hydroxypteridine-6-carboxylic acid. The latter two compounds along with riboflavin and a new pteridine were isolated from *Drosophila* by Karrer, Hadorn and associates (203, 204). They indicate their new pteridine to be most likely (-)-2-amino-4-hyroxypteridine-6-hydroxyacetic acid. Forrest & Mitchell (197) speculate on isoxanthopterin arising from uric acid since the amount of the latter in the adult fly appears negligible as compared to that present in the larval stage. Further isoxanthopterin might then be converted through a fluorescyanine-type (6-glycol?) structure to biopterin which in turn on stepwise oxidation of the side chain followed by decarboxylation could yield any one or all of the above pterins with the exception of the N^8-lactyl-derivative.

Ichthyopterin, a pteridine first isolated from fish scales by Hüttel & Sprengling (205), was considered by Tschesche & Korte (206) as 2-amino-4,7-dihydroxypteridine-6-acetic acid. However, this formulation has recently been questioned by Matsuura and co-workers (207). Their evidence indicates that ichthyopterin is an isoxanthopteryl derivative in agreement with Tschesche & Korte but that it bears an α-hydroxylated side chain. Tschesche & Barkemeyer (208) have proposed a half-acetal structure for erythropterin (VII) to account for the absence of an enediol [Purrman & Eulitz (209)] reaction.

Urothion, the only known sulfur-containing natural pteridine, isolated from human urine by Koschara some 20 years ago, has now been tentatively

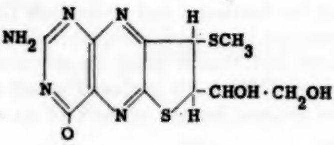


VII
Erythropterin

formulated by Tschesche, Korte & Heuschkel (210) as a fused pteridine-thiophane derivative (VIII).

The isolation of xanthopterin from human-type tubercle bacilli has been reported by Crowe & Walker (211). Burgress & Rolfe (212) isolated an unidentified crystalline pteridine from grasshopper eggs which replaced thymidine and vitamin B_{12} in growth promotion of several lactobacilli. By means of paper chromatography and bioautograms Neujahr (40) identified formyltetrahydropteroic acid in activated sewage. Two minor unidentified factors active against *Leuconostoc citrovorum* were encountered besides CF. Excellent recent summaries of advances in the biology and chemistry of pteridines have appeared (213, 214).

"Co C."—In studies on the serine-glycine interconversion Wright (215, 216) examined the cofactor requirements of an enzyme in *Clostridium HF* (217). A cell-free extract of this organism catalyzed the formation of glycine and C^{14}-formate from β-C^{14} serine. The reaction was dependent on orthophosphate and also required Mn^{++}, DPN, pyridoxal phosphate, and a new cofactor. As a source of the cofactor she employed boiled extracts of *Clostridium cylindrosporum* referring to the cofactor as "Co C." PGA, N^5-formyl PGA, and anhydroleucovorin have no activity (218); CF and $PGAH_4$ are active at very high concentrations, much higher than that necessary for Co C. The latter do not require DPN. CF was inactive with some enzyme preparations. There appears to be no relation between the DPN requirement and



VIII
Urothion

the oxidized or reduced state of the pteridine. These observations coupled with chromatographic examination of the *C. cylindrosporum* extract differentiated the new cofactor activity from FA forms earlier reported as active by Blakley (219, 220) and Kisliuk & Sakami (221, 222) in their enzyme systems. Work on a preparative scale (218, 223, 224) revealed a series of six groups of folic acid derivatives in the extract, all of which had varying amounts of Co C activity. One appeared identical with teropterin (218). Three are diglutamyl and two are hexaglutamyl derivatives of CF judging from light absorption studies and amino acid analyses. Cofactor activity appears to be related to the presence of either three or seven glutamic acid residues in the molecule rather than to the form of the pteridine. Five of the Co C fractions contain serine and glycine, two have in addition alanine and two an unidentified amino acid (224). Three of the fractions analyze for a mole of pentose, two of these in turn analyze for one mole of stable phosphate while the third analyzes for four moles of phosphate, two stable and two labile. The most potent fraction appeared to be diglutamyl CF with serine in the molecule (Co C I). The analytical data for the amino acid content other than glutamic acid indicate the Co C fractions to consist of mixed peptide species. More complete characterization of these new peptide forms thought to contain pentose and phosphate may go far in clarifying our insight into the molecular mechanics of the serine-glycine interconversion.

Synthesis of folic acid antagonists.—Spickett & Timmis (225) in an earlier study had observed a low degree of antifolic activity with 2,4,7-triamino-6-phenylpteridine, although the corresponding 7-hydroxy compound was inactive. In a continuation of this synthetic program Timmis, Osdene and their associates prepared a variety of compounds related structurally in one way or another to pteridine, e.g., 3,6-diaminopyrido-(2,3)-pyrazines (226); 3,6-diaminoquinoxalines (227); triaza-, tetraaza-, and pentaaza-1,2-benzanthracenes (228, 229) and 4,7-diamino- and 2,4,7-triaminopteridines (230). They also prepared the 4-amino-analogue of isoxanthopterin. Unfortunately, no observations on the biological effects are available.

Dick & Wood (231) reported a new synthesis of 2-amino-4-hydroxypteridines by the condensation of 2-chloro-3-methoxycarbonylpyrazines with guanidine salts. These pyrazines are available from aliphatic compounds. Older approaches (232, 233) to pteridines from pyrazines utilized pyrazine intermediates obtained by degradation of larger molecules. The new approach allows for ready isotopic labeling of the 2-position. A similar approach to 4-aminopteridines was found independently by Taylor & Paudler (234). These authors used 2-chloro-3-cyano-pyrazines with guanidine salts. Various aspects of pteridine and FA antagonist chemistry have been recently summarized (214).

Assay of folic acid.—Sobotka and co-workers (235) described a group of thermophilic bacteria, belonging to the genus Bacillus, which grow optimally at 55°C. and have a requirement for FA. One strain in particular, *Bacillus coagulans* ATCC #3084 (236), responds to all forms of FA, including con-

jugated forms, and to PABA, but not to pteroic acid. The use of one of these organisms would seem to provide a welcome advance in the technic for assaying the total FA content of tissues in which FA activity exists in the form of various conjugates which are not always efficiently hydrolyzed by present methods so that they can be accurately assayed.

Enzymatic interconversions.—Work proceeded in the attempt to identify the cofactor involved in the transfer of one-carbon units in biological processes. Zakrzewski & Nichol (237) presented evidence that the formyl carbon of CF can be derived from formate by isolating labeled CF from cells of *Streptococcus faecalis* which had been incubated with PGA and formate-C^{14}. These workers also showed (238, 239) that CF derived from PGA by bacterial or liver preparations represents the stable product which is formed under anaerobic conditions by the nonenzymatic alteration of a labile precursor which has properties similar to those of N^{10}-formyl-$PGAH_4$. Thus it would appear that the formation of CF from PGA is inhibited by the antifolics only indirectly. Sakami *et al.* reported (240) that when $PGAH_4$ is incubated in a pigeon liver extract with various other metabolites under anaerobic conditions, two products are formed, one of which is CF and another apparently identical with the labile precursor of CF described above (238).

Numerous studies were reported of *in vitro* conversions of PGA to CF and other more active forms of FA. Zakrzewski & Nichol (239) incubated PGA-triglutamate with *S. faecalis* cell extracts and produced CF-triglutamate, whereas incubating PGA with *S. faecalis* cells produced CF as well as CF di- and triglutamates. Hakala & Welch (241) showed that *Bacillus subtilis* synthesizes CF triglutamate *de novo*. Doctor & Trunnell (242) reported the enhancement by serine and formate of the conversion of PGA to CF by rat liver homogenate, and Heisler & Schweigert (243) began the purification and study of the enzyme in *Lactobacillus casei* which carries out this conversion. Couch & Reid (244, 245) also partially purified the enzyme from liver homogenate that is responsible for this conversion and found that the addition of serine, ascorbic acid, homocysteine, ATP, DPN, or Mg^{++} was stimulatory while glycine inhibited the conversion. Doctor & Trunnell (246) demonstrated that administering estradiol dipropionate with PGA to male rats promoted the conversion of PGA to CF as evidenced by an increased excretion of the latter in the urine. It was shown by Mitbander & Sreenivasan (247) that feeding chlorobutanol (Chloretone) to rats, which is known to stimulate ascorbic acid synthesis, also stimulates conversion of PGA to CF. Braganca & Kenkre (248) observed that human blood and some animal tissues when treated with trichloroacetic acid degrade PGA and 4-APGA enzymatically to a PABA derivative and a pteridine portion.

Serine and glycine interconversions.—Kisliuk & Sakami reported (221, 222) that pigeon liver extract that had been inactivated by Dowex treatment and dialysis was fully restored in its ability to convert 1-C^{14} of glycine to the 1-C^{14} of serine by the single addition of $PGAH_4$. The addition of $PGAH_4$ also

restored the ability of this inactivated extract to utilize formaldehyde, but not formate, for serine-β-carbon formation. Similar experiments were reported by Alexander & Greenberg (249) who showed that a cell-free soluble rat liver preparation catalyzed the synthesis of serine from glycine and formaldehyde. Activity was lost on dialysis but could be restored by the addition of ATP plus leucovorin or by $PGAH_4$ alone. The polyglutamyl character of the pteridine coenzyme (Co C) required in the *in vitro* conversion of serine to glycine has been noted (218, 225). Arnstein (250) studied glycine formation in the intact rat by feeding labelled L-(C^{14})alanine and D-(C^{14})glucose to normal and FA-deficient animals. He concluded that glycine biosynthesis from glucose probably involves serine as an intermediate. This same worker also demonstrated (251) that a B_{12} or ascorbic acid deficiency had no effect on the rate of glycine formation or on the glycine-serine conversion in the rat but that in folic acid deficiency the endogenous glycine production was reduced and the conversion of glycine to serine was also decreased. It was postulated by Elwyn *et al.* (252) from observed characteristics of the glycine to serine conversion in rats that the FA compound involved must be an N-5-hydroxymethyl, or a closely related, derivative of $PGAH_4$.

Greenberg, Jaenicke and co-workers (253, 254, 255) presented evidence that the actual compound involved in one-carbon transfer reactions is N^{10}-formyl-$PGAH_4$ or a closely related, more labile derivative. They believe this compound is formed in liver extracts by an ATP-dependent reaction between formate and $PGAH_4$ which is catalyzed by an enzyme they call tetrahydrofolate formylase.

Transmethylation.—Fatterpaker *et al.* (256) observed that a 0.5 per cent dietary level of nicotinamide in rats deficient in folic acid resulted in a depletion of liver choline, methionine, and phospholipides with increased neutral fat in the liver. Adding PGA or choline to the diet corrected these conditions. With baby chicks on a diet deficient in FA, Young *et al.* (257) found 0.8 per cent choline would not prevent perosis, whereas 0.05 per cent choline was sufficient for this purpose when FA was adequate. When choline was deficient the need for PGA increased from 30 μg. to 80 μg. per 100 gm. of diet. A mixture of monoethylaminoethanol and betaine was used as efficiently as choline, with or without folic acid.

Nutrition and miscellaneous.—Some less-expected effects of FA deficiency were observed. Asling *et al.* (258) noted that fetal rats from mothers deficient in FA from days 11 to 21 of the gestation period showed multiple congenital abnormalities. This same group (259) later observed similar abnormalities in fetal rats whose mothers had been given a FA-deficient diet for only 48 hr. during days 7 to 12 of the gestation period. Luckey *et al.* (260) presented evidence that the germ-free rat can synthesize more than its own need of FA provided biotin is present in its diet. Kodicek *et al.* (261) observed that rats on a FA-deficient diet containing 1 per cent succinylsulfathiazole developed multiple abcesses and infarct-like lesions in liver

and spleen. It was suggested that these lesions appeared as the result of lowered resistance toward bacterial infection, caused not only by granulocytopenia, but also by the impairment of antibody formation. Asenjo (262) also observed similar foci of infection in rats apparently deficient in FA only, without supplementation with sulfa drugs. The quantitative requirements of the "minute" streptococci for PGA and its various derivatives were reported by Felton & Niven (263). This group of bacteria show somewhat more ability than the pedicocci in transforming PGA into metabolically active forms but show a great variation among strains in their ability to use the various PGA derivatives. The activity of leucovorin for these bacteria varies from strain to strain, providing further evidence that this is not the fundamental metabolic form.

Effects of folic acid antagonists.—Interest continued in the investigation of FA antagonists which might be more useful in the treatment of the leukemias, special attention being given to the mechanism by which resistance develops to these compounds in leukemic cells. Burchenal (264) reviewed the present status of this work and called attention to the fact that so far only the 4-amino derivatives of PGA have a specific action on the acute leukemias of childhood. In his opinion further comparative studies of the mechanism of action of these compounds in bacteria, protozoa, and in normal and neoplastic tissues would be rewarding. Nichol (265) studied the ability of amethopterin-resistant intact cells of *S. faecalis* and their extracts to convert PGA to CF and concluded that the major factor involved is a marked reduction in the permeability of the bacterial cells which reduces the accessibility of the susceptible enzyme system to these compounds. Goldin *et al.* (266) developed a method for quantitatively comparing the effectiveness of FA antagonists and concluded that amethopterin is more effective than 4-APGA in the treatment of leukemia in mice. Zahl & Albaum (267) investigated the effect of FA antagonists on the adenine nucleotide levels in the tissues of tumor-bearing mice. There appeared to be a selectively greater reduction in tumor when the antagonist was given with adenylic acid. Williams *et al.* (268) observed in *in vitro* experiments that amethopterin inhibited the incorporation of formate C^{14} into the proteins and purines of leukemic mouse cells that were sensitive *in vivo* to amethopterin more than those that were resistant. There was no effect of amethopterin on the incorporation of C^{14} into the protein fraction of normal mouse liver. From similar *in vitro* studies Balis & Dancis (269) concluded that the resistance of mouse leukemia cells was the result of partial elimination of an antifolic-sensitive pathway. In studies *in vivo*, in which the amethopterin could first be acted upon by the whole animal, its effect was to reduce the incorporation of formate into thymine while increasing purine synthesis from formate. These independent and opposite effects were interpreted as indicating the existence of separate intermediates for the incorporation of one-carbon moieties into these two molecules. Metais & Mandel (270) found the DNA of the bone marrow of rats to be more sensitive than RNA to the action

of aminopterin. A 240 $\mu g.$ dose of 4-APGA repeated at three-day intervals was comparable in its effect on DNA and RNA to a 700r dose of x-rays. Administration of thymine and guanine, although not completely neutralizing the effects of the antagonist, was more effective than PGA.

Rudenberg *et al.* (271) studied FA antagonism by 1,2-dihydro-s-triazines in turnip seedlings. One of these compounds [4,6-diamino-1-(3′,4′-dichlorophenyl)-1,2-dihydro-2,2-dimethyl-s-triazine] was about 1/50 as active as 4-APGA in this system and, like the latter, was not reversed by PGA or PABA, but was partially reversed by Coenzyme I, and most effectively by CF. Inhibition by this triazine, as by 4-APGA, was attributable in part to interference with normal mitosis. It was reported by Evans & Robson (272) that 4-APGA given subcutaneously to mice produced no inhibition of the growth response of the uterus to estrogen, but when applied to the vaginal wall by implant it completely inhibited the estrogenic response. CF but not PGA reversed this effect when given by the same route. Another indication of the preferential effect of 4-APGA for certain tissues was the report by Rees *et al.* (273) that 4-APGA had a beneficial effect in 80 per cent of a small series of psoriasis cases. There was an 8 per cent incidence of toxicity, and doses had to be adjusted accurately.

Choline

Much information on choline metabolism appeared in publications primarily concerned with B_{12} and FA in their relation to the synthesis and transfer of methyl groups. Challenger (274) presented an excellent historical review of biological methylations.

In rat studies Elwyn *et al.* (252) found that the β-C^{14}, β-D, and N^{15} of labelled serine were utilized as a unit for ethanolamine synthesis, while labelled glycine and labelled formate were converted to serine prior to incorporation into ethanolamine. These three compounds were all able to furnish the carbons of the methyl groups of choline, serine being most effective. Kennedy (275) found an enzyme system in isolated rat liver mitochondria which catalyzed the incorporation of radioactive choline into phospholipides. Radioactive lecithin, isolated by chromatographic procedures, was the principal labeled product formed. Sloane *et al.* (276) demonstrated that cell-free pigeon liver extracts contain a betaine transmethylase which will synthesize methionine from homocysteine and betaine. There was no coenzyme requirement, and the reaction was specific for betaine; choline or a number of other methyl donors not being active. Both Wells (277) and Mulford (278) reported that 2-amino-2-methylpropanol-1 is a competitive antagonist of choline in the rat as measured by its ability to counteract the preventive effect of choline against renal hemorrhagic degeneration. The data were taken to indicate that this compound not only inhibits the utilization of choline in young rats but apparently also inhibits the synthesis of choline from methionine and betaine as well. Stekol *et al.* (279) presented evidence from tracer studies that in the rat choline is synthesized not by

transfer of three methyl groups of methionine to ethanolamine, but by transfer of one methyl group of methionine to dimethylethanolamine as the direct acceptor, a process not requiring FA as a cofactor although the latter is required for the *de novo* synthesis of the two methyl groups of dimethylethanolamine.

Some insight into the mechanism by which choline acts as a lipotropic factor was furnished by Artom (280), who found that tissues from rats which had received choline shortly before they were sacrificed oxidized fatty acids more rapidly than controls. Since choline *in vivo* enhanced the oxidation of fatty acids in the isolated liver, whereas addition of choline *in vitro* was ineffective, it was postulated that the effect was attributable to some complex substance formed from choline *in vivo* but not *in vitro*. This active factor also appeared to be formed in extrahepatic tissues, such as the kidney and heart, but at a slower rate.

Two pathways for the biosynthesis of lecithin have been assumed to exist in mammalian liver preparations. By one pathway free choline is incorporated into the lecithin molecule by a reaction not involving the intermediate formation of phosphorylcholine, but requiring ATP and CoA. Phosphatidyl CoA has been suggested as an intermediate. By the other pathway phosphorylcholine is incorporated as a unit into lecithin. Kennedy and Weiss (281) have reported that this second pathway requires cytidine-5′-triphosphate as a catalyst, but not CoA or ATP.

Several workers continued the attempt to find if there is a correlation between dietary choline and cardiovascular conditions in experimental animals. Knudson & Harris (282) reported that in rats fed a choline-free diet for five days after weaning, followed by seven months on a choline-supplemented diet, only 3 per cent showed blood pressure readings over 124 mm. Hg, while in control rats fed continuously on a choline-free diet 34.7 per cent had an elevated blood pressure. The animals with high blood pressure showed no significant changes in cholesterol concentration in blood, heart, kidneys, or liver. Baxter & Goodman (283) attempted to confirm the claim of Wilgram *et al.* (284) that choline exerts a "lipotropic" effect not only in the liver and kidneys but also in the cardiovascular system. They studied the renal changes in young rats on low choline diets and found that the fatty deposits and necrosis in the kidneys occurred practically simultaneously, and it was not clear whether the renal fatty changes were a cause or a manifestation of injury. More recently Wilgram *et al.* (285) reported that if cholesterol is added to the diet of rats which are already deficient in choline, cardiovascular lesions appear whereas they do not appear in choline-deficient rats not receiving cholesterol. Pilgeram (286) believes there is a definite correlation between the ability of different animal species to form phosphatidylcholine from free ethanolamine and their relative resistance to experimental atherosclerosis. Burns & Ackerman (287) reported that a choline deficiency in the laying hen resulted in smaller eggs which had

a lower concentration of fat and choline. Supplementation with either choline or B_{12} counteracted these deficiencies.

p-*Aminobenzoic acid.*—A thermophilic bacterium, *Bacillus stearothermophilus*, reported by Sobotka *et al.* (235) to require FA as a growth factor, could utilize PABA as a substitute, and the effect of the latter was counteracted by sulfonamides. Dubnoff (288) isolated a series of *E. coli* strains with varying B_{12} requirements from the original *E. coli* mutant 113-3. The strain with the lowest B_{12} requirement responded submaximally to PABA, which had no effect on the highest B_{12}-requiring strain. Purko *et al.* (289) reported that *E. coli* cells which were grown in media containing PABA had an enhanced ability, compared to cells grown in the absence of PABA, to carry out hydroxymethylation of α-ketoisovalerate in the synthesis of pantothenate. This hydroxymethylation reaction was completely inhibited by sulfonamides and competitively reversed by PABA, but not by PGA or CF. Braganca & Kendre (248) found an enzyme in blood and animal tissues which degrades PGA to PABA derivatives and pterins. Rollo (290) pointed out that strains of *Plasmodium gallinaceum*, the malarial parasite, which have become resistant to proguanil and pyrimethamine, still remain sensitive to the sulfonamides. This is presumably attributable to the fact that the former two drugs act by interfering with the conversion of FA to CF while the sulfonamides interfere with the utilization of PABA by the parasite.

The authors of this review agree with the authors of last year's review (4) that PABA should not be considered a vitamin since its action is confined to the metabolism of microorganisms and it bears no relation to the nutrition of animal species.

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WATER-SOLUBLE VITAMINS, PART II[1,2]

(VITAMIN B_6, NICOTINIC ACID, BIOTIN, ASCORBIC ACID)

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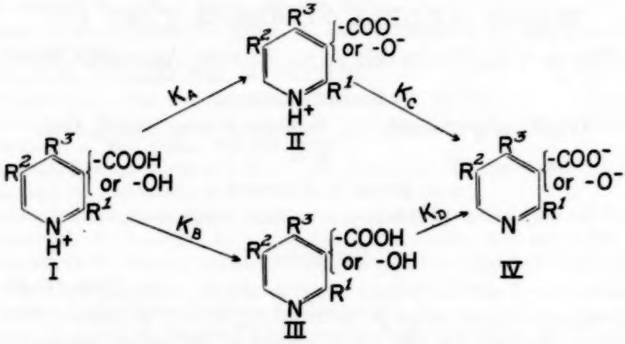
VITAMIN B_6

Chemistry.—A novel synthesis of pyridoxine by rearrangement of an appropriately substituted furan is described by Elming & Clauson-Kaas (1). Preparative methods for two cyclic acetals of pyridoxine, isopropylidenepyridoxine and cyclohexylidenepyridoxine (2), for several pyridoxylidene derivatives of various amino acids (3) and amines (4), and for pyridoxamine-5-phosphate by direct phosphorylation of pyridoxamine with metaphosphoric acid (5) have been patented. Each of these products has vitamin B_6 activity for one or more organisms.

For the complete understanding of biological function, the dissociation constants of acid groups and the relative amounts of various ionic forms of a vitamin present in solution are important. Even for compounds as well known as pyridoxal and nicotinic acid, such information has not been available. Such data have now been reported for nicotinic acid and 3-hydroxypyridines including vitamin B_6. With these compounds, as with amino acids (6), the most acidic form (I) upon titration with base can dissociate either to a dipolar ion (II) or to a neutral uncharged form (III). Further titration leads to IV. Dissociation constants K_A, K_B, K_C, and K_D are assigned as indicated. These are related to the experimentally observed first and second dissociation constants, K_1 and K_2, as follows: $K_1=K_A+K_B$ and $1/K_2=1/K_C+1/K_D$. For alpha amino acids, K_B is so small that K_1 is essentially equal to K_A, and the neutral form of the amino acid consists virtually entirely of the dipolar ion. However, in the aminobenzoic acids K_A and K_B are nearly the same, and the isoelectric compounds are mixtures of dipolar ions and uncharged molecules (6). Several years ago Harris, Webb & Folkers showed that isoelectric pyridoxine consists largely of dipolar ions (7). Recently, Metzler & Snell have studied 3-hydroxypyridine and various forms of vitamin B_6 spectrophotometrically (8). Apparent values for pK_1 and pK_2 of 5.10 and 8.60 were obtained for 3-hydroxypyridine. Substantial amounts of both the dipolar ion (54 per cent) and uncharged molecules (46 per cent) were present in the isoelectric solution. These data are sufficient to compute $pK_A=5.37$,

[1] The survey of the literature pertaining to this review was completed in November, 1955.

[2] The following abbreviations are used in this chapter: DPN for diphosphopyridine nucleotide; INH for isonicotinyl hydrazide.



Nicotinic acid: R^1, R^2, R^3 = H; COOH present
Pyridoxine: $R^2 = CH_2OH$ $R^3 = CH_2OH$, $R^1 = CH_3$; OH present
Pyridoxal: $R^2 = CH_2OH$, $R^3 = CHO$, $R^1 = CH_3$; OH present
Pyridoxamine: $R^2 = CH_2OH$, $R^3 = CH_2NH_3^+$, $R^1 = CH_3$; OH present

$pK_B = 5.44$, $pK_C = 8.33$, and $pK_D = 8.26$. These values are supported by subsequent calculations of Jaffé (9), who extended use of the Hammett equation to pyridine derivatives and predicted values for pK_A, pK_B, and pK_D of 5.42, 5.30, and 8.54, respectively, in good agreement with the experimental values. The corresponding constants for pyridoxal, pyridoxamine, and pyridoxine are derivable from data of Metzler & Snell, who also estimate the percentages of nondipolar ion forms of vitamin B_6 in isoelectric solutions as follows: pyridoxine, 12 per cent; pyridoxal, 8 per cent; and pyridoxamine monohydrochloride, 3 per cent (8). Experimental values for the dissociation constants of vitamin B_6 are available from this study (8), that of Williams & Neilands (10), and that of Lunn & Morton (11). The latter workers also emphasize the dipolar ionic character of these compounds.

The values of pK_1 and pK_2 of nicotinic acid have been determined spectrophotometrically by Evans *et al.* (12) and by Hakala & Schwert (13) as 2.09, 2.10 and 4.88, 4.75, respectively. Jaffé confirmed these data titrimetrically (9), and calculated (via the Hammett equation) that for nicotinic acid $pK_B = 3.3$, $pK_C = 4.7$, and $pK_D = 3.6$. It follows that pK_A is about 2.2 and that the isoelectric form is again largely the dipolar ion with about 5 per cent of the uncharged form present. In accordance with this view, the pK of trigonelline (nicotinic acid methyl betaine), which should be similar to that of pK_A (and pK_1) for nicotinic acid, is 2.10 (13); and the pK of nicotinamide, which should approximate the pK_B for nicotinic acid, is 3.10. The situation with isonicotinic acid is similar (9, 12, 14, 15). Thus, correlation of the dissociation constants for these vitamins and related compounds with the vast amount of data on other pyridine and benzene derivatives through the Hammett equation is quite satisfactory.

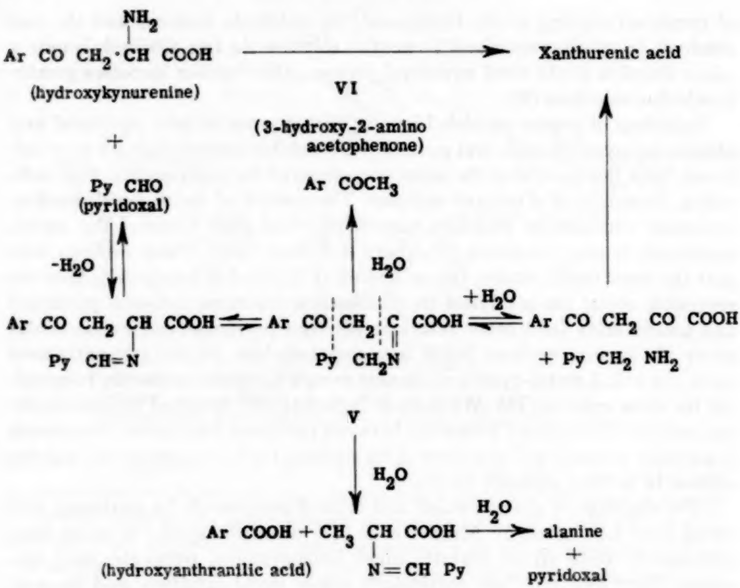
Spectrophotometric data permit an estimate of the relative proportions

of pyridoxal existing as the hemiacetal, the aldehyde hydrate, and the free aldehyde in aqueous solutions. In neutral solution the free aldehyde is only a minor fraction of the total pyridoxal present; this fraction increases greatly in alkaline solutions (8).

Solutions of copper or nickel ions in the presence of both pyridoxal and alanine (or pyridoxamine and pyruvic acid) exhibit spectra that are very different from the spectra of the metal complexes of the components, thus indicating formation of a ternary complex. The spectra of metal-pyridoxamine-pyruvate solutions on standing become identical with those of the metal-pyridoxal-alanine complexes [Eichhorn & Dawes (16)]. These findings support the postulate [Metzler, Ikawa & Snell (17); Fried & Lardy (18)] that the reversible metal ion-catalyzed transamination reactions between pyridoxal and amino acids (and other reactions between pyridoxal and amino acids) occur via an intermediate Schiff base-metal-chelate. At the concentrations used, the 1:2:2 metal-pyridoxal-alanine complex appears primarily responsible for these spectra (16). Williams & Neilands (10) detected titrimetrically azomethine (Schiff base) formation between pyridoxal and amino compounds in aqueous solution and also showed formation of a 1:2 copper-pyridoxamine chelate in neutral aqueous solutions.

The cleavage of cystathionine and related compounds by pyridoxal and metal salts first noted by Metzler *et al* (17) proceeds rapidly at room temperature to yield (from cystathionine) homocysteine, pyruvate, and ammonia. Cu^{++} and Zn^{++} are particularly active metal catalysts, and the rate of the reaction is substantially increased by addition of appropriate chelating agents [Binkley (19)]. In the presence of pyridoxal and metal salts, kynurenine undergoes transamination to yield pyridoxamine and kynurenic acid; apparently the *o*-aminobenzoylpyruvic acid first formed undergoes spontaneous ring closure [Longenecker & Snell (20)]. Cleavage of kynurenine to anthranilic acid and alanine was not observed in the model system. The latter reaction occurs enzymatically and may be visualized in either of two ways: (*a*) as an α,β-elimination followed by oxidoreduction between the resulting *o*-aminobenzaldehyde and α-aminoacrylic acid-pyridoxal-metal complex to yield anthranilic acid and alanine (20), or (*b*) as cleavage of a β-diketone-like intermediate [Braunshtein & Shemyakin (21)], as illustrated below for hydroxykynurenine. Indirect evidence consistent with the former mechanism was found in model systems by Longenecker & Snell; the latter mechanism (21) is consistent with findings of Dalgliesh (22), who detects small amounts of 2-amino-3-hydroxyacetophenone (VI, Ar=2-amino-3-hydroxyphenyl) in urine of vitamin B_6-deficient animals fed tryptophan. He visualizes its occurrence as resulting from cleavage of V, an intermediate analogous in structure to an α,β-diketone, which occurs in the transamination reaction (17, 18), and which by hydrolysis yields VI. A similar hydrolytic cleavage at the equivalent position between the γ and β carbon atoms of V would yield alanine and hydroxyanthranilic acid.

Enzymology.—In accordance with activity of pyridoxal in model reac-



tions (17, 23) and in growing bacterial cultures (24), pyridoxal phosphate is an essential coenzyme in the interconversion of serine and glycine by purified enzyme preparations from mammalian livers [Blakley (25); Alexander & Greenberg (26)]. In each case serine is converted reversibly to glycine and formaldehyde. No evidence of activation by metal ions [required in the model reaction (23)] was obtained (25); in some pyridoxal-dependent enzymic reactions, the apoenzyme may fill the role played by metal in the model systems (17, 25). In view of the avidity with which proteins bind some metal ions, however, their possible role in the enzymic reactions can scarcely be eliminated until they are shown to be absent in the pure enzyme. Pyridoxal phosphate is reportedly the coenzyme for a crystalline enzyme from crayfish muscle, citrulliniminase, that converts citrulline and ammonia to arginine (27).

A single enzyme from mammalian livers appears to decarboxylate cysteinesulfinic acid and cysteic acid, the former much more rapidly [Hope (28); Fromageot *et al.* (29)]. Activity of this enzyme in rats decreased to very low levels after only two to three days on a vitamin B_6-deficient diet; during this period, the activity of liver extracts is greatly enhanced by pyridoxal phosphate. After more than four days on the deficient regimen, the apoenzyme disappears from liver, but not from brain (29). Cysteine desulfhydrase activity of liver also decreases within a few days after withdrawal of vitamin

B_6 [Thompson & Guerrant (30)]. This sensitivity of enzymes concerned in cysteine metabolism to vitamin B_6 deficiency led Snell (31) to suggest that excretion of sulfur compounds following a test load of cysteine be examined as a possible index of vitamin B_6 insufficiency in man.

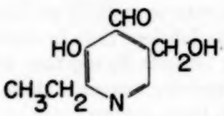
Decarboxylation of diaminopimelic acid by *Escherichia coli* (32), transamination of D-amino acids by *Bacillus subtilis* (33), and the combined transamination and deamidation that glutamine and asparagine undergo with various keto acids (34, 35) are additional processes shown within the past year to be catalyzed by vitamin B_6 proteins.

Vitamin B_6 antagonists.—Isonicotinyl hydrazide (INH) administration to human patients causes excretion of an excess of vitamin B_6 in the urine [Biehl & Vilter (36)], possibly as the isonicotinyl hydrazone of pyridoxal [synthesized by Sah (37)], and administration of pyridoxine with INH prevents the peripheral neuritis which otherwise occurs in 60 per cent of the patients. Rosen (38) and Boone *et al.* (39) note that toxic symptoms produced by INH in rats are more pronounced in vitamin B_6-deficient than in normal animals; such symptoms are prevented by feeding vitamin B_6 together with INH. Although some of the symptoms of INH toxicity resemble those of uncomplicated vitamin B_6 deficiency, others do not. Epileptiform convulsions are more frequent in INH-treated animals than in control animals. While acrodynia is mild on the vitamin B_6-deficient diet and is pronounced in animals fed deoxypyridoxine, it is not observed in INH-fed animals (40). Similarly, xanthurenic acid excretion is not enhanced and may be slightly lessened by feeding INH; it is greatly increased by 4-deoxypyridoxine (38). Thus, although both substances act as antagonists of vitamin B_6, they seem to affect a different assortment of reactions. That these effects of INH are produced via inhibition of pyridoxal-dependent enzymes seems beyond question, as indicated by (*a*) the increased excretion of pyridoxal in INH-fed patients (36), (*b*) the observed decreases in the level of some vitamin B_6-enzymes in tissues that follow INH administration [e.g., glutamine and asparagine transaminases (35)], and (*c*) the observed competitive action of INH in preventing activation of certain cell-free, pyridoxal-dependent enzymes by pyridoxal phosphate [e.g., apotryptophanase (18, 40)]. Even so, there is no evidence that the therapeutic action of INH in tuberculosis results from a similar action, for both in mice [Greenberg & Blencowe (41)] and guinea pigs [Brun *et al.* (42)] administration of pyridoxine with INH does not affect the antituberculous activity of the drug. Cymerman-Craig *et al.* (43) and Gangadharam & Sirsi (44) note the pronounced chelating ability of INH and suggest that it may function by withdrawing essential heavy metals from enzyme systems such as the transaminase (43, cf. 17) or iron-porphyrin systems (44).

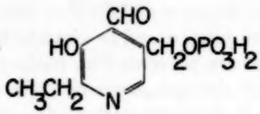
The antivitamin B_6 action in rats and mice of the pyrimidine portion of thiamine [toxopyrimidine, Makino (45)] has been confirmed [Abderhalden (46)]; pyridoxine, pyridoxal, and pyridoxamine all counteract its effects. Toxopyrimidine phosphate (2-methyl-6-amino-5-(hydroxymethyl)pyrimi-

dine-5-phosphate) inhibits the action of tyrosine decarboxylase from *Streptococcus faecalis*; its action is antagonized by pyridoxal phosphate [Makino & Koike (47)].

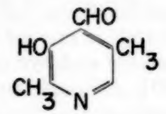
Under conditions such that synthesis of D- from L-alanine by the alanine racemase is the growth-limiting reaction in *S. faecalis*, Olivard & Snell (48) found that ω-methylpyridoxal (VII) was about 3.0 per cent as active as pyridoxal in promoting growth at low L-alanine concentrations, but about 10 per cent as active at high concentrations of L-alanine. Correspondingly, ω-methylpyridoxal phosphate (VIII) fully activated the cell-free alanine aporacemase but only at concentrations 10 times those of pyridoxal phosphate. The affinity of the VIII-activated racemase for alanine was only about half that of the pyridoxal phosphate-activated racemase. 4-Deoxypyridoxine does not inhibit growth of *S. faecalis* under these conditions; cor-



VII



VIII



IX

respondingly, neither 4-deoxypyridoxine nor 4-deoxypyridoxine phosphate inhibited the alanine racemase when added simultaneously with pyridoxal phosphate. 5-Deoxypyridoxal (IX), however, inhibited growth and the cell-free alanine racemase (48).

By appropriate omissions from the growth medium, synthesis of any one of several amino acids by vitamin B_6-dependent enzymes was made growth-limiting. Depending upon the limiting amino acid (and hence the limiting enzymic reaction within the cell), the effects of ω-methylpyridoxal (VII) ranged from efficient substitution for pyridoxal as a growth factor, to those of a competitive inhibitory analogue of pyridoxal. Similarly, ω-methylpyridoxal phosphate replaced pyridoxal phosphate as coenzyme for several cell-free enzymes involved in amino acid metabolism (e.g., alanine racemase, phenylalanine-glutamic transaminase, and others), but failed to replace it for others, e.g., cysteine desulfhydrase [Olivard & Snell (49)].

These experiments and others cited elsewhere (25, 31) demonstrate that the affinities of various enzymes of a single organism for a common coenzyme (in this case pyridoxal phosphate) differ, and do not necessarily parallel their affinities for analogue coenzymes (e.g., ω-methylpyridoxal phosphate). This is illustrated in the following equations in which let A, B, etc., represent a series of different vitamin B_6-dependent apoenzymes of a given organism, and K_1, K_2, etc. be equilibrium constants representing the affinities for coenzyme or inhibitor (similar arguments will apply if the measured "affinities" actually represent reaction rate differences). Then, in general, K_1 is not equal

$$A + \begin{cases} \text{pyridoxal phosphate} \overset{K_1}{\rightleftharpoons} \text{holoenzyme A (functional)} \\ \text{analogue coenzyme} \overset{K_2}{\rightleftharpoons} \text{protein A (nonfunctional)} \end{cases}$$

$$B + \begin{cases} \text{pyridoxal phosphate} \overset{K_3}{\rightleftharpoons} \text{holoenzyme B (functional)} \\ \text{analogue coenzyme} \overset{K_4}{\rightleftharpoons} \text{protein B (nonfunctional)} \end{cases}$$

to K_3 and K_1/K_2 is not in general equal to K_3/K_4. Thus diet-induced deficiencies of vitamin B_6 in different animal species need not, in general, result in the same enzymic deficiencies or in the same deficiency symptoms, and in a given animal all of the vitamin B_6-dependent enzymes will not decrease in activity at the same rate. Similarly, analogue-induced vitamin deficiencies generally do not (cf. 38, 39, 50, 51, 52) and should not be expected to duplicate deficiencies produced by deficient vitamin intakes, for the primary sites affected may be quite different in the two cases, and the observed differences in symptomatology do not provide evidence against the view that the analogue acts solely as a vitamin antagonist, as has sometimes been assumed (50). This view is in accord with results with INH-fed rats cited earlier; with the observations of Dietrich & Shapiro (51) that enzymic deficiencies [and symptomatology (cf. 50)] in 4-deoxypyridoxine-fed rats do not parallel exactly those in vitamin B_6-deficient rats; and with the observations of Sandman & Snell (52) that symptoms produced in rats by ω-methylpyridoxine differed from those observed in vitamin B_6-deficient control animals. The latter analogue has significant growth-promoting action in rats, but the survival time is reduced as compared to deficient controls, and convulsions occur with greatly increased frequency (52).

Umbreit (50) has reviewed the mode of action of 4-deoxypyridoxine. Certain abnormalities in tryptophan metabolism induced by azaserine and 6-mercaptopurine are corrected by simultaneous administration of pyridoxine [Magill *et al.* (53)].

Effects of vitamin B_6 in animals.—Renal phosphate-activated glutaminase, but not the similar enzyme from liver, is significantly lowered in vitamin B_6-deficient rats [Beaton *et al.* (54, 55)] and restored to normal (in homogenates) by addition of pyridoxal phosphate. In liver, the pyruvate-activated glutaminase is significantly lowered during B_6 deficiency (55). These observations may correlate with the subnormal blood glutamine and supernormal blood glutamic acid in deoxypyridoxine-fed, B_6-deficient rats [Beaton *et al.* (56)]. Reduction in the asparagine and glutamine transaminases of rat tissues by INH administration and restoration by pyridoxal phosphate has also been noted by Meister (35). Fasting levels of blood sugar and liver glycogen are reduced after five days of vitamin restriction; after three weeks blood levels of inorganic phosphate and glutathione were increased [Beaton & Goodwin (57, 58)]. Further details of the effects of B_6 deficiency in rats

on excretion of intermediates and derived products in the tryptophan-nicotinic acid conversion are supplied by Henderson, *et al.* (59) and Dalgliesh (60).

In contrast to rats, dogs exhibit no increase in blood urea during vitamin B_6 deficiency, and no increased urea production follows test loads of amino acids [Hawkins & Young (61)]. Feeding deoxypyridoxine to B_6-deficient dogs produces conjunctivitis with mucous discharge, blepharitis, some denudation, and skin lesions similar to those observed by Vilter in man. All except denudation improve rapidly on supplementation with vitamin B_6 [Hawkins (62)].

Daily treatment of rats with 50 mg. of vitamin B_6 significantly reduces severity of, and shortens recovery time from the hemolytic anemia induced by injection of serum from dogs injected with washed rat erythrocytes (63). Antibody response in rats to diphtheria toxoid is reduced in B_6 deficiency (64).

On a basal diet deficient in both vitamin B_6 and vitamin E, rats excrete greatly increased amounts of creatine and allantoin and have an elevated xanthine oxidase. Supplementation of the diet with either vitamin prevents these changes. The specific activities of excreted creatinine and creatine following administration of formate-C^{14} were equal, indicating that this type of nutritional muscular dystrophy in the rat results from inability of the muscle to retain creatine [Dinning *et al.* (65, 66)].

Any of a variety of amino acids (including norleucine and norvaline) partially replace vitamin B_6 as a growth factor for a clone of excised tomato roots [Boll, (67)]. Glycine and serine were most effective and could be replaced by ethanolamine; in the latter instance, appropriate vitamin B_6 assays showed a true sparing effect on vitamin B_6. The author interprets these effects as indicating a general role of vitamin B_6 in amino acid synthesis and in production of choline (via ethanolamine) from serine, perhaps by decarboxylation.

Clinical.—Two reviews (68, 69) discuss the role of vitamin B_6 in human nutrition. Wager (70) contributes an additional report of the helpful effects of pyridoxine in control of nausea and vomiting of pregnancy.

Coursin (71) reports a series of cases showing that continued suboptimal dietary intakes of less than 60 μg. of vitamin B_6 per liter of milk produce disturbances of the central nervous system in infants. This figure agrees well with other assays (69) of milk formulas that permit development of convulsions. Administration of pyridoxine to 31 children with cryptogenic and secondary epilepsy was of no value (72).

Increased intakes of pyridoxine in both monkeys and man lead to an increased blood level of glutamic-alanine transaminase and of vitamin B_6 itself [Marsh *et al.* (73)]. Serum transaminase also increases markedly in 90 per cent of cases of myocardial necrosis and within 36 hr. after myocardial infarction, but not in the myocardial ischemia of angina pectoris (74). The finding may be of diagnostic value. Schroeder (75) discusses the possible role of vitamin B_6 deficiency in production of atherosclerosis in man.

Combinations of pyridoxine and raw linseed oil provide an effective treatment for phrynoderma (76). Previous reports of absorption of pyridoxine through the skin are confirmed by Villela (77).

Palmer (78) reports that intravenous pyridoxine is effective in treatment of acute alcoholism and delirium tremens. However, Small *et al.* (79) and Gruber (80) find no favorable effects in acutely alcoholic subjects.

Assay of vitamin B_6.—Heat sterilization of liquid milk products decreases their vitamin B_6 content as indicated both by microbiological and rat assay; greater losses were indicated by the latter procedure [Tomarelli *et al.* (81)]. The two methods give similar results with spray-dried milk samples. Use of a mutant culture of *E. coli* for vitamin B_6-assay is recommended by Diding (82). Although the method appears adequate for products high in vitamin B_6, interference in the minimal medium employed by amino acids of samples low in the vitamin seems certain from the relatively high growth activity displayed by a vitamin-free casein hydrolysate.

Nicotinic Acid

Nutrition.—Attempts are being made to place the nicotinic acid-tryptophan relationship on a quantitative basis. Horwitt (83) suggests that in man 4.7 mg. of nicotinic acid per 1000 kcal. prevents pellagra, but 3.9 mg. does not. Tryptophan is about 1/60 as active by weight as nicotinic acid. The high corn diets consumed by Guatemalan Indians do not produce pellagra despite the pellagragenic properties of certain corn diets because they contain, on the average, 500 grams per day of corn providing an estimated 8.8 mg. of nicotinic acid and 228 mg. of tryptophan, the equivalent of about 5.0 mg. of nicotinic acid per 1000 kcal. If Horwitt is correct, such a diet would not cause pellagra, but if about half of the corn were replaced with sweet potatoes, corn syrup, starch or other carbohydrate food, pellagra would result. Thus, corn is not exonerated as the culprit in human pellagra, for it is hard to conceive of any pellagra-causing diet adequate in protein except one based on corn.

Goldsmith *et al.* (84) conclude that a daily intake of 8 to 10 mg. nicotinic acid equivalents per day is adequate to prevent pellagra on diets of 1600 to 2100 kcal., i.e., about 4.9 mg. per 1000 kcal., in accord with Horwitt. However, with diets deficient in nicotinic acid, pellagra developed more slowly on a "wheat" than on a "corn" diet, and less N^1-methyl nicotinamide was excreted on the former diet. The possibility that corn contains a "pellagragenic" factor thus is not ruled out, but the effect of such a factor must be relatively small.

One alternative explanation of the "pellagragenic" nature of a corn diet postulates an amino acid imbalance that decreases conversion of tryptophan to nicotinic acid. Thus supplementation of a 10 per cent casein diet with gelatin or oxidized casein increases the tryptophan requirement for satisfactory growth by 30 per cent [Sauberlich & Salmon (85)]. Similarly, rats receiving a purified amino acid diet containing only 0.1 to 0.11 per cent tryptophan develop nicotinic acid deficiency when the remaining amino acids are fed at levels optimum for growth on other rations. A decrease in the leucine, iso-

leucine, valine, threonine, or lysine content improves growth because of a less severe nicotinic acid deficiency [Henderson & Koeppe (86)]. A complete description of the nicotinic acid nutrition of man will have to consider this factor [review (87)] and also the occurrence of the vitamin in nutritionally unavailable forms. The nicotinic acid of corn and other cereals, for example, is largely present in a "bound" form unavailable to pigs, rats, and chicks [Braude *et al.* (88)]. If the same is true for man, the pellagragenic effect of maize is easier to understand. The bound vitamin becomes available on mild hydrolysis, and the importance of lime treatment in liberating the vitamin in corn used in central American diets, although still uncertain, appears probable (89).

A diet for evaluation of nicotinic acid nutrition in man furnishes 10 mg. of nicotinic acid and 1 gm. of tryptophan daily. The 24 hr. excretion of N^1-methylnicotinamide and its 6-pyridone are measured. Nicotinic acid-deficient subjects excrete very little of these metabolites. Two weeks or more on the diet permit a more precise evaluation. Depleted subjects require many days to reach normal excretion levels; well-nourished patients reach the maximum excretion rate rapidly [Goldsmith *et al.* (90)]. By this evaluation procedure, patients with diabetes mellitus appear to contain normal nicotinic acid stores. In contrast, alloxan-diabetic rats excrete abnormally low amounts of N^1-methyl nicotinamide [McDaniel *et al.* (91)]; on insulin treatment, this returns to normal.

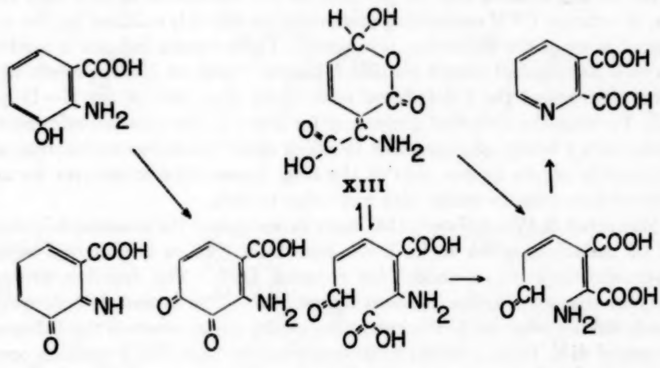
The DPN (diphosphopyridine nucleotide) level of red blood cells appears an adequate guide to assessment of dietary nicotinic acid deficiency in rats [Burch *et al.* (92)]. Human red cells contain much less DPN, and the method may not be applicable to man. A low intake of nicotinamide or its precursors leads to a decreased level of liver pyridine nucleotides and serum N^1-methylnicotinamide in rats. However, Fisher *et al.* (93) find no correlation between the liver pyridine nucleotide level and growth rate.

Acute nicotinic acid deficiency in calves has been studied by Hopper & Johnson (94). Germ-free White Leghorn chicks on a nicotinic acid-free diet died after about four weeks; conventional birds continued to gain with little mortality. Excreta of the germ-free birds contained amounts of the vitamin sufficient if ingested to prolong the birds' lives [Lucky *et al.* (95)].

Biosynthesis.—The conversion of tryptophan to nicotinic acid also occurs in peas (96) and in Rhesus monkeys (97). When dietary nicotinic acid is adequate, the tryptophan undergoing conversion is largely converted to quinolinic acid [Banerjee & Basak (97)]. In man L-tryptophan raises excretion of nicotinic acid metabolites markedly the first day; D-tryptophan acts at a much slower rate [Tomabechi (98)]. Heretofore there has been no conclusive evidence for the conversion of tryptophan to nicotinic acid in most bacteria. Yanofsky (99) now finds that neither isotopically labeled indole nor tryptophan is converted to nicotinic acid by *B. subtilus* or by *E. coli*, and growth of three nicotinic acid auxotrophs of *B. subtilis* is not supported by any of the known intermediates in the tryptophan-nicotinic acid conversion. Ap-

parently an alternative method of nicotinic acid synthesis occurs in these organisms.

Some details of the mechanism of conversion of tryptophan to nicotinic acid are still uncertain. 3-Hydroxyanthranilic acid (but not its amide) is a precursor of nicotinamide in rats, but the amount of conversion is low [Henderson *et al.* (100)]. A slight conversion of anthranilic acid to 3-hydroxyanthranilic acid apparently occurs, insufficient to maintain growth of rats. A prominent side reaction in the conversion of 3-hydroxyanthranilic acid to nicotinic acid is the formation of quinolinic acid. When this reaction is catalyzed by a rat liver enzyme preparation [Schweigert *et al.* (101, 102); Long *et al.* (103); Mehler, (104, 105)], an unstable intermediate with an absorption maximum at 360 mμ in neutral solution is produced by an enzymic oxidation which requires ferrous iron. This is readily converted to quinolinic acid nonenzymically. A second enzyme converts it into a compound spectroscopically similar to, but not identical with, nicotinic acid (104). Structures X, XI,



X. XI XII XIV

and XII are among those suggested for the labile intermediate. Structure X (102) apparently is excluded, since the intermediate is in the same oxidation state as quinolinic acid (103). While substance XI may be an intermediate, Hayaishi, *et al.* (106) feel that a single enzyme oxidizes and opens the ring. Structure XII in contrast to the intermediate, would react with carbonyl reagents and is certainly unstable with respect to other related structures because of severe steric hindrance to formation of a planar conjugated system. The lactol, XIII, although not previously suggested, has many of the necessary characteristics of the labile intermediate. Subsequent conversion to Substance XIV would precede ring closure to quinolinic acid. Nicotinic acid could result by enzymic decarboxylation of XIV.

3-Acetylpyridine is converted to nicotinic acid in dogs and is about one-sixth as active as nicotinic acid in increasing excretion of N[1]-methylnicotini-

mide [McDaniel *et al.* (107)]. The compound is at the same time a nicotinamide antagonist as discussed previously (18). In ducklings, β-picoline, pyridyl-3-carbinol, and pyridyl-3-aldehyde replace nicotinamide and are converted into pyridine nucleotides [Van Reen & Kaplan (108)]. Pyridyl-3-carbinol is as effective as nicotinic acid and nicotinamide in maintaining the level of DPN in red blood cells (92).

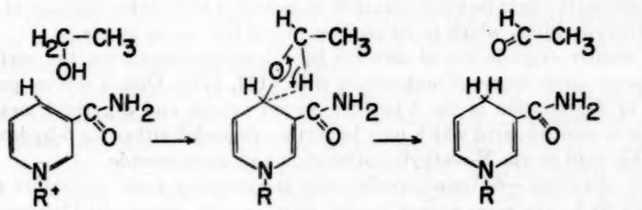
Catalytic mechanisms.—Position 4 of the pyridine ring is the site of enzymic hydrogen transfer (109, 110). These transfers are stereospecific. Alcohol dehydrogenase, lactic dehydrogenase, and malic dehydrogenase (111) transfer hydrogen to and from one side of the ring (side 1) whereas a β-hydroxysteroid dehydrogenase (112) and pyridine nucleotide transhydrogenase (113) from *Pseudomonas* transfer hydrogen from the opposite side (side 2). A striking preference for a particular side of the ring in nonenzymic oxidations also exists. Both isomeric mono-deutero-reduced DPN have been prepared. If the reduced DPN containing deuterium on side 1 is oxidized with neutral ferricyanide, about 50 per cent of the deuterium is removed. However, if reduced DPN containing deuterium on side 2 is oxidized by the same reagent, none of the deuterium is removed. These results indicate a combination of a preferential attack on side 1 together with an isotope effect which makes cleavage of the C—H bond more rapid than that of the C—D bond (113). Perhaps the observed preference for side 1 in the nonenzymic reaction results from a folded configuration in which steric hindrance to reaction with ferricyanide occurs on one side of the ring. Reasonable structures for such hindered forms can be made with molecular models.

Mauzerall & Westheimer (114) have investigated the nonenzymic reduction of malachite green to its leuco base by 2-, 4-, or 6-deutero-1-benzyl-dihydronicotinamide, a model for reduced DPN. The reaction proceeds slowly in ethanol solution at room temperature. The 2- and 6-deutero-compounds did not transfer deuterium to malachite green, whereas the 4-deutero-compound did. Thus, a direct hydrogen transfer from the 4-position occurs in the model system as in the enzymic oxidations of reduced DPN. The reaction resembles the enzymic transhydrogenase system but is not very similar to the enzymic reduction of carbonyl compounds. A number of other reductions (e.g., of alloxan and quinone) were carried out by 1-methyl-, 1-propyl-, 1-tetracetylglucosyl-, and 1-benzyl-1, 4-dihydronicotinamide.

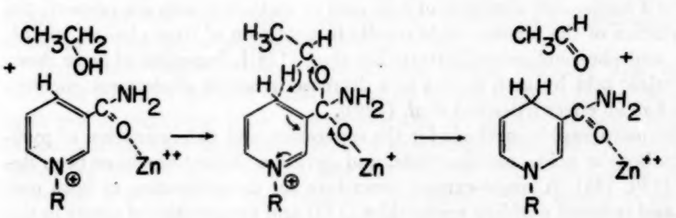
On reduction of DPN by the irradiation of oxygen-free ethanolic solutions a product is obtained which, like reduced DPN, absorbs light at 340 mμ and reduces methylene blue, but unlike reduced DPN does not fluoresce or act as a coenzyme for lactic or alcohol dehydrogenase [Swallow (115)]. The compound is probably DPN reduced at the 2- or 6-positions. This is in contrast to the reduction by dithionite which leads to an enzymically active compound reduced at the 4-position. The same type of experiment with 1-methylated and 1-propylated nicotinamides gives similar results (116, 117). Electrolytic and irradiation reductions involve radical mechanisms and yield

a different product than that obtained by the ionic mechanisms operating in enzymic and dithionite reductions (115, 117, 118).

Kaplan has reviewed the mechanisms involved in DPN oxidations (119). Burton & Kaplan (120) have shown that many nucleophilic reagents add at the 4-position of DPN. They suggest that in alcohol dehydrogenase action an ethoxide ion may add to DPN after which the complex breaks down to give acetaldehyde and reduced DPN (see A). The four-membered ring in the suggested transition state seems unlikely, however, and other plausible mechanisms can be visualized. For example, alcohol might react first with another portion of the enzyme such as the amide group of nicotinamide, as illustrated in B. A hydride transfer with formation of reduced DPN and acetaldehyde would then occur. Such a process might be greatly assisted by the participation of the chelated zinc ion of alcohol dehydrogenase (121) as shown in B.



A



B

Catabolism.—Using bioautographic techniques with *Lactobacillus arabinosus*, Van Eys, Touster & Darby (122) demonstrate existence of a new metabolite of nicotinic acid in rat urine, tentatively identified as β-nicotinyl-D-glucuronic acid.

When INH (50 mg. per 100 gm.) is included in the diet of rats, no growth suppression, depletion of liver pyridine nucleotides, or decrease in excretion of N[1]-methylnicotinamide was observed, either following injection of L-tryptophan or during a control period if the diet contained adequate vitamin B_6 [Rosen (38); see also vitamin B_6 section]. INH apparently does not act primarily as a nicotinic acid antagonist.

Many strains of *Pseudomonas fluorescens* utilize nicotinic acid as a sole source of carbon and nitrogen; formation of the oxidative system is adaptive [Hughes (123)]. Such organisms do not metabolize 2-hydroxynicotinic, 2,6-dihydroxynicotinic, 5-bromonicotinic, or 6-fluoronicotinic acids, but do oxidize 6-hydroxynicotinic, 5-chloronicotinic, 5-fluoronicotinic, and 2-fluoronicotinic acids. These results suggest that the pyridine ring is opened at carbon 6 rather than at carbon 2, and the isolation of 6-hydroxynicotinic acid during the early stages of the degradation of nicotinic acid supports this view. 5-Chloronicotinic acid yields a compound thought to be 5-chloro-6-hydroxynicotinic acid. Since 5-chloro and 5-fluoronicotinic acids are further oxidized, formation of 5,6-dihydroxynicotinic acid is not a likely further step, and Hughes concludes that the 6-hydroxy derivative is opened to form a glutaconic dialdehyde derivative which is further oxidized. Formation of this glutaconic dialdehyde derivative from 6-hydroxynicotinic acid involves a reduction; it might be more plausible to assume a hydrolytic cleavage of the 6-hydroxypyridine, which in its pyridone form is a cyclic amide.

A similar degradation of nicotine by microorganisms from the surface of tobacco seeds occurs [Frankenburg *et al.* (124, 125)]. One of several pathways of degradation is via 3-pyridyl propyl ketone and 3-pyridyl methyl ketone to nicotinic acid which may be further degraded either via 6-hydroxynicotinic acid or via N-methylnicotinamide and nicotinamide.

Miscellaneous.—6-Aminonicotinamide is extremely toxic to rabbits and appears to be the most potent known nicotinamide antagonist [Johnson & McColl (126, 127)]. One-half per cent nicotinamide in the diet may be toxic for rats if inadequate amounts of folic acid or methyl donors are present; the methylation of the nicotinamide results in depletion of liver choline, methionine, and phospholipides [Fatterpaker *et al.* (128)]. Ingestion of large doses of nicotinic acid by man results in a decrease in serum cholesterol; nicotinamide has no effect [Altschul *et al.* (129)].

Chromatographic methods for the separation and determination of pyridine carboxylic acids, nicotinamide, and pyridine nucleotides have been devised (130, 131). A single-extract procedure for determination of both oxidized and reduced pyridine nucleotides (132) and two studies of errors in the fluorimetric determination of pyridine nucleotides and N^1-methyl nicotinamide have been reported (133, 134). Methyl ethyl ketone is claimed to be superior to acetone in the fluorimetric procedure (92).

Biotin

Thirty-eight of 46 coagulase negative strains of staphylococci had an absolute requirement for biotin, and the remaining 8 strains achieved maximum growth only when biotin was present [Niven *et al.* (135)]. In contrast, 90 coagulase positive strains grew without added biotin. Biocytin (ϵ-N-biotinyl-L-lysine) was equally as active as biotin for the 18 strains tested; dethiobiotin replaced biotin for 17 of these 18 cultures. Homobiotin also replaced biotin for many but not all of these cultures (135).

Sixty-six of 69 strains of the pathogenic yeast, *Candida*, all of human origin, required biotin for growth [Drouhet & Couteau (136)], as do all strains tested of the pathogenic fungus, *Blastomyces dermatitidis* (137).

According to Lichstein (138), the dose-response curve of *Propionibacteria* to biotin differs from that shown by other bacteria in showing a linear turbidity increase with arithmetic (rather than with logarithmic) increases in biotin concentration. Use of *Propionibacterium pentosaceum* as an assay organism indicated occurrence of undefined changes in biotin on long-continued refrigeration or on autoclaving with the medium. These changes were not detected by assay with yeast or *L. arabinosus*. All of the propionibacteria tested utilized dethiobiotin or oxybiotin in place of biotin (138).

Micrococcus sodonensis requires biotin but is nearly one hundred times more sensitive to it than *L. arabinosus* and may therefore be valuable as an assay organism. Dethiobiotin replaces biotin, a disadvantage if a specific assay is desired. Interestingly, biocytin is about 165 per cent as active as biotin on the molar basis [Aaronson (139)]. A modified yeast assay method for biotin is described by Fillipov (140).

Biotin stimulates or is essential for growth of many strains of *Mycobacterium tuberculosis* grown in oleic acid-albumin agar; for some of these, the biotin requirement is eliminated by culturing under increased CO_2 tension [Schaefer *et al.* (141)]. This sparing effect of CO_2 on the biotin requirement is similar to that earlier described for lactic acid bacteria and for *Clostridium butylicum*, where carbon dioxide also must be supplied in media containing both oleic and aspartic acids to permit growth without added biotin [Broquist & Snell (142)], and probably reflects the still undefined role of biotin in fixation or production of CO_2 or both.

Little additional light has been thrown during the past year on the utility of biotin in the diet of animals or on its metabolic function. Separate additions of methylene blue, neutral red, and particularly ascorbic acid to a biotin-deficient diet suppressed clinical symptoms of biotin deficiency and prolonged the life span of the deficient animals, although not to normal [Terroine, (143)]. These agents did not maintain normal weight or nitrogen balance and did not prevent the increase in urinary ammonia found in deficient animals. Sundaram *et al.* (144) showed previously that biotin antagonists block nicotinic acid synthesis in *Neurospora* and pea seedlings and that this effect was overcome by biotin. Dalgliesh (60) now finds that the excretion pattern for kynurenine, hydroxykynurenine, xanthurenic acid, anthranilic acid, and their conjugates following a test dose of tryptophan is unchanged from normal during severe biotin deficiency. The role of thiamine, riboflavin, and vitamin B_6 is readily revealed by a change in this pattern during deficiencies of these vitamins; thus, no evidence of a role of biotin in the transformation of tryptophan to nicotinic acid in animals was obtained.

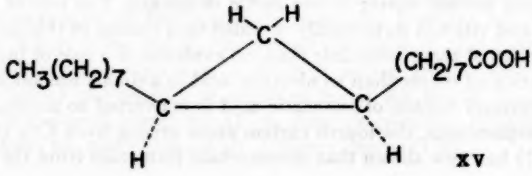
The isopropyl residue of isovaleric acid is converted to acetoacetate by rat liver preparations, the fourth carbon atom arising from CO_2 (145, 146). Fisher (147) has now shown that acetoacetate formation from the β-methyl

acids, isovaleric, 3-methyl-2-butenoic, and 3-methyl-3-butenoic by normal rat liver mitochondria is dependent upon added CO_2. Mitochondria from biotin-deficient animals do not form acetoacetate even when CO_2 is present; this ability was restored by injections of biotin for three days prior to killing. Acetoacetate formation from *n*-valeric and *n*-caproic acids proceeded normally in both types of animals (13). The finding again illustrates the role of biotin in CO_2-fixing reactions *in vivo*. The observation [Sarma *et al.* (148)] that riboflavin synthesis by *Aspergillus oryzae* is greatly decreased by biotin sulfone or 2-(3,4-ureylenecyclohexyl)butyric acid, and restored in their presence by biotin, may have a similar explanation; for CO_2 and purine bases are known precursors of riboflavin (149), and incorporation of CO_2 into purine bases by animals also decreases in biotin deficiency [MacLeod & Lardy (150)]. Despite this essential role in purine base formation, biotin depresses synthesis of ribo- and deoxyribonucleic acids by two lactobacilli and by *B. subtilis* (151); a less pronounced effect is given by aspartic acid and Tween 80.

In contrast to previous results with oxalacetate decarboxylase from varied sources (152), Lichstein (153) finds biotin in hydrolysates of an oxalacetic decarboxylase concentrate from chick liver. The report does not provide evidence for the association of the biotin with the enzyme, since the latter was not highly purified, and only about one part of biotin was present per 10^9 parts of protein.

A number of components of nutrient media spare biotin for bacteria such as *L. arabinosus*. These include aspartate, unsaturated fatty acids, acetate, and CO_2 (142), and in the absence of aspartate, threonine, lysine, and uracil (154). Similarly, the inhibitory effects of biotin sulfone on growth are alleviated by aspartic acid and oleic acid, and in the presence of moderate but suboptimal concentrations of aspartic acid by threonine, lysine, and uracil [Ravel & Shive (155)]. The effects of the latter could only be duplicated by very high concentrations of aspartate, which therefore serves as a precursor for these three compounds in *L. arabinosus*. Thus biotin is, at least indirectly, essential for synthesis of these amino acids and plays many functional roles in metabolism.

The effect of unsaturated fatty acids in sparing or, under appropriate conditions, eliminating the requirement of many bacteria for biotin has been frequently mentioned in this and past reviews (142, 156). Niven *et al.* (135) showed that Tween 80 (a nontoxic source of oleic acid) replaced biotin for 17



XV

of 18 staphylococcal strains when added to a hydrolyzed casein medium. Hofmann and co-workers (157, 158) have shown lactobacillic acid (XV) to be a major component of lipides of *L. arabinosus* and *Lactobacillus casei*; other cyclopropane fatty acids are known to occur naturally (159). Hofmann & Panos (159) now report that lactobacillic acid, in contrast to straight chain saturated fatty acids, but like many unsaturated fatty acids, replaces biotin for many lactic acid bacteria and approaches these in activity. The activity of several related acids for various bacteria is compared (159). In a subsequent paper [Hofmann *et al.* (159a)] the complete fatty acid spectrum of several lactic acid bacteria is reported. Unsaturated fatty acids equal or exceed lactobacillic acid in concentration in all species examined, and in some of these, lactobacillic acid is low relative to the unsaturated fatty acids. Determination of the shift in fatty acid composition of such cells following growth with individual active fatty acids replacing biotin in the medium should do much to clarify the possible role of biotin in synthesis of unsaturated fatty acids and lactobacillic acid, and the biosynthetic relationships between the unsaturated fatty acids and the cyclopropane fatty acids.

The natural occurrence of an antimetabolite of biotin, actithiazic acid [(-)-2-(5-carboxypentyl)-4-thiazolidione], in culture filtrates of *Streptomyces* should be recorded (160). According to Nysterakis (161) the inhibitory effect of auxin on the growth of *Nectria galligena* is counteracted by biotin.

In early studies leading to recognition of pimelic acid as a biotin precursor, Eakin & Eakin (162) showed that both biotin and cysteine increased biotin production by *Aspergillus niger*. Wright and co-workers find more biotin-l-sulfoxide than biotin is produced when either dethiobiotin (163) or pimelic acid (164) is added as precursor. No stimulation in production of biotin plus biotin sulfoxide was noted on addition of cysteine; however, biotin production was increased, probably because the presence of cystine protects a portion of the biotin from oxidation to the sulfoxide. Thus, no evidence for cysteine as a biotin precursor yet exists. Azelaic acid and pimelic acid were equally active in stimulating biotin formation; suberic and sebacic acids were inactive. Oleic acid, which yields azelaic acid as one product on oxidative scission of the double bond, was ineffective; its effectiveness in other organisms in sparing of biotin appears not to be attributable to action as a precursor. All evidence points to degradation of azelaic to pimelic acid (β-oxidation) prior to its action as a biotin precursor (164).

Ascorbic Acid

Biosynthesis.—The direct conversion of D-glucose into L-ascorbic acid via D-glucurono-γ-lactone and L-gulono-γ-lactone, and the analogous conversion of D-galactose appears established in both plants and animals (18). The enzyme from pea seedlings that catalyzes reduction of D-galacturonic acid methyl ester, probably to L-galactono-γ-lactone, is found in the soluble part of the cytoplasm; the reducing agent is reduced triphosphopyridine nucleotide [Mapson & Isherwood (165)]. The previously investigated en-

zyme which converts L-galactono-γ-lactone to L-ascorbic acid was found in the mitochondria (166).

An alternative pathway starting with L-sorbose has been tested by Burns *et al.* (167). Chlorobutanol (Chloretone)-treated rats converted uniformly labelled L-sorbose-C^{14} to urinary ascorbic acid to the extent of only 0.3 per cent during 24 hr. following its injection. When C_6-labeled L-sorbose was given, about half of the C^{14} in the isolated ascorbic acid was in carbon atoms 1 and 6, while the remainder was distributed in carbons 2 through 5. Thus L-sorbose was not converted by a direct oxidation to 2-keto-L-gulonic acid and thence to ascorbic acid, but presumably was fragmented, converted to glucose and then to ascorbic acid.

Cabbage leaves, germinating seeds, and lemon juice have been examined with inconclusive results for the possible presence of reductones intermediate in ascorbic acid formation [von Euler & Hasselquist (168)]. The relation between ascorbic acid formation in seedlings and photosynthesis has been investigated by Sugawara (169). Ascorbic acid synthesis in plants and its relation to carbohydrate metabolism [Franke (170)] and synthesis of ascorbic acid in higher animals have been reviewed [Cedrangolo (171)].

Functions.—Despite the mass of data relating to the effects of ascorbic acid in enzyme systems and intact organisms, nothing certain is known of the exact function played by this vitamin.

Eagle (172) cultures human embryonic and cancer cells on a highly defined medium without addition of ascorbic acid. Most other water-soluble vitamins are required in the medium. Small amounts of dialyzed serum are essential which may possibly contain small amounts of ascorbic acid. Thus despite presence of relatively high concentrations of this vitamin in tissues, any intracellular requirement for it must be small.

Apparently the best case for a specific role for ascorbic acid can be made for the hydroxylation of *p*-hydroxyphenylpyruvic acid to homogentisic acid [Knox (173)]. The renal enzyme catalyzing this reaction requires small amounts of ascorbate. Glucoascorbic and isoascorbic acids are also active but may be poorly retained in the body and hence inactive *in vivo*. Dichlorophenolindophenol or hydroquinone also activate the enzyme *in vitro*. Although it may be premature to call ascorbic acid a coenzyme for this reaction, it probably represents one specific site of ascorbate function (173).

Model hydroxylation reactions involving ascorbic acid and ferrous iron [Udenfreund *et al.* (174)] were reviewed last year (18). The same system has been extensively studied by Dalgliesh (175) with similar results. Anthranilic acid, kynurenine, tryptophan, and indoleacetic acid are hydroxylated in positions *ortho* or *para* to the nitrogen attached to the ring, i.e., at electronegative sites. Phenylalanine is converted to tyrosine, and tyrosine to 3,4-dihydroxyphenylalanine and hydroquinone. The latter reaction involves the displacement of the side chain to form alanine or serine. The side chain can be removed from phenylacetic acid and β-phenylethylamine in a similar manner. These reactions probably proceed via a quinol type of intermediate

analogous to that believed to occur during enzymic hydroxylation of *p*-hydroxyphenylpyruvic acid. In the latter enzymic reaction, rearrangement to 2,5-dihydroxyphenylpyruvic acid occurs (173, 176), but in the model reactions so far studied the side chain splits off. The reagent permits preparation of such unstable compounds as catechols and *o*- and *p*-aminophenols without difficulty.

Torii & Moriyama (176) claim that in the presence of ascorbic acid and air, tryptophan can be converted to kynurenine, and they cite a claim by Imanoga that the decomposition of histidine by aerobic iron-ascorbic acid mixtures [Edelbacher & von Segesser (177)] leads to formation of aspartic acid, apparently paralleling the biological degradation of histidine by *Pseudomonas* (178). Incubation of long-chain fatty acids carboxyl-labeled with C^{14} with excess ascorbic acid aerobically at 38°C., pH 7 yields $C^{14}O_2$, apparently by oxidative reactions [Geyer *et al.* (179); Ottolenghi *et al.* (180)]. Possible biochemical implications are discussed.

Dalgliesh (175) suggests that a free radical might be the attacking reagent in the model hydroxylation system. Monodehydroascorbic acid has often been suggested as such an agent. However, in most known reactions, enzymic or otherwise, ascorbic acid is associated with either copper or iron, and the reactions may center around a metal chelated with ascorbic acid; free radicals in the usual sense may not participate.

Apparently the hydroxylating agent in these model systems arises during the course of ascorbic acid autooxidation in the presence of suitable metals. Nord has recently investigated kinetically the autooxidation of ascorbic acid in the presence of cuprous chloride in hydrochloric acid solution (181). The basic observations of earlier workers were confirmed, and a mechanism involving free radicals was suggested. Kinetic constants for the various steps were evaluated and from these the steady-state distribution of copper between the +1 and +2 oxidation states was computed and found to be in good agreement with the ratio as measured spectrophotometrically.

Nason *et al.* (182) and Kern & Racker (183) show that systems from peas and yeast that oxidize reduced DPN are activated by ascorbic acid in the presence of ascorbic acid oxidase or cupric or ferric iron salts. Apparently some intermediate formed during ascorbic acid autooxidation is required here as in the model hydroxylating systems.

A special role for ascorbic acid in metabolism of the cell nucleus is postulated by Stern & Timonen (184); ascorbic acid, glutathione, and pyridine nucleotides are all present. Ascorbic acid catalyses electron-transport between reduced DPN and the cytochrome system in the adrenal glands of pigs. In whole homogenates, dehydroascorbic acid is partially effective; in a mitochondrial preparation only ascorbic acid is effective. The authors suggest that the active form possesses an oxidation state intermediate between ascorbic and dehydroascorbic acids [Kersten *et al.* (185)].

Although a marked stimulation of photosynthetic phosphorylation of ascorbic acid occurs either aerobically or anaerobically in washed spinach

chloroplasts [Arnon *et al.* (186, 187)], there is no conclusive evidence in favor of a role for ascorbic acid *in vivo* in either oxidative phosphorylation or photosynthetic phosphorylation. Wessels (188) has studied the photooxidation of ascorbic acid by isolated chloroplasts and by alcoholic solutions of chlorophyll *a*.

Takeda & Hara (189) have recently reiterated the opinion (190) that the primary function of ascorbic acid is in mobilization of ferrous iron. Ascorbic acid is thought to reduce ferric iron of ferritin to ferrous iron [cf. also Mazur *et al.* (191)] and then to transfer it to aconitase, homogentisicase, and other ferrous iron-requiring enzymes. Support for this idea was found in experiments with young guinea pigs (189) in which either ascorbic acid deficiency or α,α'-dipyridyl injection led to similar defects in operation of the citric acid cycle. Alterations in steroid metabolism in ascorbic acid deficiency may be related to errors in the citric acid cycle [Bacchus & Lampkin (192)].

In two rhesus monkeys with chronic ascorbic acid deficiency the serum iron level fell to about one-third of normal, and the animals became anemic. Daily oral doses of 50 mg. of iron (as ferrous sulfate) failed to raise the serum iron level and increased the hemoglobin only moderately. Combined therapy with iron and ascorbic acid markedly increased both serum iron and hemoglobin levels [Greenberg & Rhinehart (193)]. Similarly, administration of ascorbic acid to human subjects with low serum iron and hemoglobin levels results in a rise in these levels [Bagchi & Chowdhury (194)]. The observations support a role for ascorbic acid in iron mobilization. An interesting reciprocal relationship is suggested by findings of Kruchakova (195) that simultaneous intake of ascorbic acid plus iron salts results in a higher level of tissue ascorbic acid in the guinea pig than does ingestion of ascorbic acid alone.

Continuing studies with purified enzymes confirm the idea that ascorbate has no specific role in homogentisic acid oxidation. Homogentisic acid oxidase requires ferrous iron, and ascorbate activates the enzyme, apparently by protecting the iron [Crandall (196)]. Knox & Edwards (197) conclude that the native enzyme contains bound iron.

Catabolism.—In man, guinea pigs, and rats part of the label of ascorbic-1-C^{14} acid appeared in respired CO_2 and part in the urine as free ascorbic acid, 2,3-diketogulonic acid, and oxalate [Burns (198, 199); Curtain & King (200)]. L-Ascorbic acid-6-C^{14} also gives rise to labelled CO_2, so the whole chain must be extensively degraded. Injection of dehydroascorbic acid into guinea pigs raised the tissue ascorbate level and urinary excretion of degradation products. Dehydroascorbic acid did not appear in the tissues or urine, and large doses were toxic. In man, small doses behave much like ascorbic acid, 3 mg. of dehydroascorbic acid being equivalent to 1 mg. of ascorbic acid [Clayton *et al.* (201)]. Ascorbic acid oxidase of barley roots is associated with the cell walls, confirming previous reports [Honda (202)]. An atypical ascorbic acid oxidase occurs in the slime mold, *Physarum polycephalum* [Ward (203)].

Miscellaneous.—Acid degradation of dehydroascorbic acid yields 3-hydroxy-5-methyl tetronic acid, whereas weak alkaline treatment also leads to decarboxylation with the production of two additional reductones [von Euler & Hasselquist (204, 205)]. The effect of ascorbic acid and other reductones on the viscosity of pectin solutions has been investigated (206). Ascorbic acid reacts with alloxan to form dehydroascorbic acid and alloxantin (207), as it does with ninhydrin to form hydrindantin (208). The dehydroascorbic acid produced is slowly decarboxylated to L-xylosone, though dehydroascorbic acid alone does not decarboxylate.

In agreement with Sumerwell & Sealock (209), Jeffay (210) found as much as 25 per cent of liver ascorbic acid of adult rats and pigs in a form not extractable from the protein by cold ethanol. Shamrai (211) has described a polarographic method for determination of a combined form of ascorbic acid detected chromatographically in peppers and dog rose fruit.

Ascorbic acid increases urinary excretion of citrovorum factor in man [Parsons *et al.* (212)] and greatly enhances conversion of folic acid to citrovorum factor by cell-free extracts of *Lactobacillus casei* [Heisler & Schweigert (213)]. In view of the importance of this conversion [review (214)] to functioning of folic acid, ascorbic acid deficiency should act as a mild "antifolic acid" agent of possible value in cancer treatment [Miller & Sokoloff (215)]. Liposarcomas grew more slowly in guinea pigs on an ascorbic acid-deficient diet and were destroyed by less irradiation than in control animals. Definite benefits in the treatment by irradiation of some tumors in human patients resulted from ascorbic acid depletion.

Ascorbic acid and various enediols are apparently not toxic to bacteria, but diketones formed from them are [Myrvik & Volk (216)], especially to species of *Mycobacterium.* Intraperitoneal injection of 100 to 200 mg. of ascorbic acid per day for six weeks into guinea pigs was without toxic effects [Lambden & Schweiker (217)].

The uptake of radioactive sulfate by mucopolysaccharides of connective tissue (granulation tissue) in guinea pigs is markedly lowered by ascorbic acid deficiency, and collagen formation is impaired [Kodicek & Loewi (218); cf. Reddi & Norstrom, in (18)].

A statistically significant decrease in serum ascorbic acid and increase in dehydroascorbic acid occurs in patients with meningococcal meningitis, tetanus, acute lobar pneumonia, typhoid fever, and tubercular meningitis [Chakrabarti & Banerjee (219)]. These toxic manifestations may possibly result, in part, from dehydroascorbic acid (219).

Formation of cataracts in senility may result from a decrease of ascorbic acid in the lens [Kulezycka (220)]. Lenses from elderly persons with yellow, brown, or black cataracts contained from a trace to 6.2 mg. of ascorbic acid per 100 gm. as compared to 28 mg. per 100 gm. in the lenses of normal young persons. When lenses were incubated *in vitro* color changes occurred which could be prevented by ascorbic acid.

Tonzig & Trezzi (221) believe that in plants indoleacetic acid functions

only as a protein complex, formation of which is competitively prevented by ascorbic acid. Arrigoni & Marrè report an inhibition of enzymic ascorbic acid oxidation *in vitro* by auxins (222).

Several papers deal with the role of ascorbic acid in adrenal function (223 to 226). The relationship between ascorbic acid intake, serum concentrations, general health, and economic status of large numbers of healthy persons over 50 years of age (227) and the serum concentrations and excretion of ascorbic acid on various dietary ascorbic acid levels in women (228) have been studied.

An extensive histological and histochemical study of skeletal abnormalities in scorbutic guinea pigs has appeared [Van Wersch (228a)]. The alkaline phosphatase activity of bone is markedly decreased, and there is a great increase in glycogen content. The author considers scurvy in man and guinea pig as a skeletal disease characterized by hypofunction of the osteoblasts, in contrast to rickets, where a hyperfunction of the osteoblasts occurs (228a).

Several modified methods for determining ascorbic acid or dehydroascorbic acid or both have appeared (229 to 236). Three of these are chromatographic procedures (234, 235, 236).

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WATER-SOLUBLE VITAMINS, PART III[1,2]

(Pantothenic Acid, Inositol, Riboflavin, Thiamine, Lipoic Acid (Thioctic Acid), Unidentified Factors)

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Pantothenic Acid

Certain strains of rumen lactic acid bacteria showed a growth response to pantethine and a further slight stimulation by trypsin-hydrolyzed casein. A response was also obtained with CoA, but pantothenic acid could not replace pantethine (1). *Lactobacillus bifidus* of avian origin showed a growth response to fish solubles. Up to 60 per cent of the growth response to fish solubles could be replaced by pantethine alone and the remainder by a factor in hydrolyzed casein. The growth stimulation by hydrolyzed casein could not be replaced by vitamin B_{12}, orotic acid, or lipoic acid (1).

The growth effect of pantothenic acid and pantothenic acid derivatives was tested with several protozoa (*Tetrahymena pyriformis, Colpidium campylum, Glaucoma scintillans*). Pantothenic acid was more active than its conjugated forms for *T. pyriformis* and *C. campylum.* Thus pantothenic acid was five to seven times more active than pantethine, *dl*-4′-phosphopantetheine, or CoA in the case of *Tetrahymena* (2). It was reported previously that *Tetrahymena* was unable to utilize CoA for growth (3). However, at that time CoA was tested only at levels corresponding to the pantothenic acid requirement. It is apparent now that these levels were too small since *Tetrahymena* requires levels of CoA above 1 μg. per ml. for growth response (2). In contrast to *Tetrahymena* and *Colpidium*, pantethine is more active

[1] The material in this review was selected from papers appearing before November 1, 1955. Because of limitation of space it was not intended to make the coverage all-inclusive, but rather to emphasize some areas of investigation. Thus certain areas have been neglected.

[2] The following abbreviations are used: ACTH for adrenocorticotropic hormone; AMP for adenosine-5′-phosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); FAD for flavin-adenine-dinucleotide; FMN for riboflavin-5′-phosphate; OYE for old yellow enzyme; P*i* for orthophosphate; PP for inorganic pyrophosphate; TPN for triphosphopyridine nucleotide; TPNH for triphosphopyridine nucleotide (reduced form).

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than pantothenic acid for *Glaucoma scintillans*. Pantothenol or pantoyl lactone are inactive for these protozoa even when β-alanine is present (2).

A favorable effect of pantothenic acid and CoA on the growth of epidermis tissue and tissue cultures has been reported (4). Growth studies with cultures of chick embryo fibroblasts and with myoblasts revealed that the most favorable concentration of pantetheine is one-tenth that of pantothenic acid. While pantetheine stimulated the growth of the cell cultures markedly at 67 μg. per ml., it exerted a toxic effect at 830 μg. per ml. (5). The toxicity of pantetheine has been explained on the basis of a possible hydrolysis to pantoic acid and cysteamine; the latter was previously shown to be toxic to such tissue preparations (6). Biesele (7) also observed a beneficial effect of CoA on the development of tissue cultures of embryonic mouse skin and Crocker mouse sarcoma 180. In such preparations mitosis was blocked by 6-mercaptopurine; the inhibition could be relieved by the addition of CoA. The mitochondria of 6-mercaptopurine treated cells became thin and fragmented; in the presence of CoA they were longer and thicker. CoA caused an accumulation of lipide droplets in the cytoplasm of embryo skin fibroblasts while 6-mercaptopurine inhibited lipogenesis. This inhibitory effect of 6-mercaptopurine was partially counteracted by CoA. The experiments with tissue cultures suggest that 6-mercaptopurine acts as an antimetabolite of CoA (7).

The feeding of a pantothenic acid deficient diet to breeder turkey hens resulted in a marked reduction of hatchability; however, egg production was unaffected even at the lowest level of pantothenic acid fed. About 16 mg. of pantothenic acid per kg. of ration was found to be required for optimum hatchability (8). Stothers *et al.* (80) obtained optimum growth and feed efficiency of baby pigs with approximately 12.5 mg. calcium pantothenate per kg. of "synthetic" milk diet. The observation of Daft (9,10) that the addition of ascorbic acid to diets low in pantothenic acid lessened the severity of pantothenic acid deficiency in the rat has been confirmed by Everson *et al.* (11). It was estimated that the protective effect of 2 per cent ascorbic acid in the diet was equal to about 100 μg. pantothenic acid. It seems possible that the synergistic effect of pantothenic acid and ascorbic acid could be explained by enhanced reducing conditions in the cell, leading to greater availability of the metabolically active thiol form of CoA.

Dinning, Neatrour & Day (12, 13) have observed an interrelationship between the pantothenic acid and methionine requirements of the rat. When rats were raised on a diet supplying the bulk of the nitrogen as soybean protein in the presence of adequate pantothenic acid, a supplement of 0.1 per cent of the diet of methionine resulted in normal levels of blood lymphocytes. In the absence of dietary pantothenic acid, a supplement of 1.73 per cent methionine was required to maintain normal levels of lymphocytes (12). When the basal diet was supplemented with either pantothenic acid or methionine, an increase of CoA per unit weight of liver was observed (13). Supplementation of the diet with both methionine and pantothenic

acid led to a further increase in the CoA level. The effect of methionine might be attributable to sparing of cystine, since Chernick *et al.* (14) found that methionine as well as cystine stimulated growth and elevated the liver CoA content of rats fed a *Torula* yeast diet. However, they obtained no effect on liver CoA upon the administration of protective amounts of vitamin E and found no consistent correlation between the level of CoA of liver and necrotic liver degeneration in animals fed the *Torula* yeast diet (cf. 15). Boxer *et al.* (79) found that the concentration of CoA in liver and kidney, but not in brain, of vitamin B_{12} deficient rats was higher than in normal animals or in pair-weighted controls.

Grob *et al.* (16) obtained an increased production of carotenoids by *Mucor hiemalis* when pantothenic acid or pantethine was added to the growth medium with acetate as the only carbon source. Ovaries from cod in the first stages of regeneration were found to have a high pantothenic acid content (1650 μg./gm. of dry weight) (17). These values are larger than those previously reported for the royal jelly of bees (18). The pantothenic acid content of tropical foods has been summarized (19).

The formation of taurocholic acid from cholic acid and taurine has been studied in rat liver homogenate systems (20). A mixture of microsomes and particle-free supernatant solutions from such homogenates carried out the conjugation reaction when ATP was present (21). Microsomes alone were inactive; however, the activity was restored upon the addition of boiled particle-free supernatant and ATP. The investigations of Elliott (22) and Bremer (23) suggest that cholyl-CoA is an intermediate in the synthesis of conjugated bile acids, since they found that the formation of cholylhydroxamic acid from cholic acid and hydroxylamine proceeded optimally with microsome preparations in the presence of CoA and relatively high levels of ATP.

Stern (24) was able to replace certain of the acyl-CoA compounds of the fatty acid cycle with the corresponding pantetheine derivatives. Evidence was also presented for exchange reactions between acylpantetheine derivatives and CoA. Crotonyl-patetheine reacted much more slowly than the CoA derivative in the presence of crystalline crotonase. Acyl-pantetheines were entirely inactive for crystalline citrate condensing enzyme and succinyl-CoA-acetoacetate transferase; pantetheine did not substitute for CoA with acetokinase and butyrokinase.

Bean *et al.* (25, 28, 29) administered a purified diet containing ω-methylpantothenic acid to four human volunteers [see also Anonymous (26); Waife (27)]. Severe clinical symptoms developed on this regime which could not be reversed simply by the administration of pantothenic acid (4 gm. daily) but required the feeding of a "good" general diet supplemented with oral and parenteral vitamins. Also, the development of symptoms suggestive of adrenal insufficiency required emergency therapy with cortisone. A decreased excretion of acetylated *p*-aminobenzoic acid has been observed by several investigators in pantothenic acid-deficient rats (30, 50). In the

experiments with the four subjects receiving ω-methylpantothenate (28) this expected pattern of urinary excretion was approached in one case; in the others it was quite erratic. The effect of ω-methylpantothenate treatment on gastric secretion and motility in humans was also examined (29). No significant change in gastric motility could be observed. In two out of three cases gastric secretion was depressed on this regime; secretion was restored upon termination of the experiments. However, in view of the special measures required for the recovery period it is difficult to judge if the re-enhanced secretion can be attributed to the effect of pantothenic acid. The response to the insulin test for gastric volume did not completely return to normal during the experimental period. The technical difficulties of conducting such experiments with human subjects are understandable, and it has not yet been possible to demonstrate a clear cut deficiency of pantothenic acid in man. Bird *et al.* (31) were unable to produce a pantothenate deficiency with ω-methylpantothenic acid (ω-methylpanthethine was also inactive) in rats. This is in contrast to the antimetabolite-metabolite relationship between ω-methylpantothenic acid and pantothenic acid previously reported for rats and mice (32, 33) and the observation that rats receiving ω-methylpantothenate had a decreased ability to acetylate sulfanilamide, suggesting that ω-methylpantothenic acid was antagonistic to some function or functions of pantothenic acid in animals (30). Drell & Dunn (34) discovered the competitive relationship between ω-methylpantothenic acid and pantothenic acid in many species of lactobacilli which required this vitamin. Bird *et al.* (31) have examined the inhibitory effect of ω-methylpantothenic acid and ω-methylpantethine[4] for several microorganisms. In general, the inhibition of ω-methylpantothenate was more effectively counteracted by pantothenate and that of ω-methylpantethine by pantethine. However, the type of inhibition varied with the organism employed; thus, a competitive relationship was found between ω-methylpantethine and pantethine with *Lactobacillus helveticus* while in *Leuconostoc citrovorum* the inhibition by the former is noncompetitive with pantothine but competitive with pantothenic acid. Pantothenylaminoethane and pantothenylaminoethanol were better antagonists of pantothenic acid in microorganisms than the corresponding doubly altered analogue ω-methylpantothenylaminoethane and ω-methylpantothenylaminoethanol (31). Pantothenylaminoethane and pantothenylaminoethanol had pantothenate activity for the rat. αα-Dimethyl pantothenic acid was prepared. It was found to be a competitive inhibitor of pantothenic acid in *Lactobacillus arabinosus* 17–5, inhibition index = 10,000 (35).

Basford & Huennekens (36) examined commercial preparations of CoA by paper and column chromatography. Four distinct forms were observed; all of them were active in the presence of cysteine or reduced glutathione

[4] Different lots of ω-methylpantothine were found to vary considerably in inhibitory activity (31). The most effective preparation was employed in these studies.

in the enzymatic assays used. One of the forms is CoA while two derivatives lack free sulfhydryl groups and appear to contain a disulfide linkage. The fourth derivative reacts more slowly with nitroprusside or 2,6-dichlorophenolindophenol than CoA. The compound does not appear to be an acyl-CoA since the hydroxylamine-ferric chloride test is negative. On the basis of Calvin's observations (37) with glutathione it is proposed that this form of CoA is a thiazoline, formed by an intramolecular reaction between the carbonyl group of the terminal amide linkage and the thiol group of CoA. In the case of the proposed thiazoline form of CoA the spectral region of thiazoline is unfortunately obscured by that of adenine, and to the present time proof for this grouping rests on the rates of reaction with nitroprusside and 2,6-dichlorophenolindophenol which correspond to those of certain other thiazoline compounds (38). It is not certain whether the different forms of CoA observed arise during the preparation (39) of CoA or whether they occur in natural products.

Investigation of the effect of pantothenic acid nutrition on adrenal-cortical function has continued. Deb *et al.* (40) and Hurley & MacKenzie (41) found levels of liver glycogen and blood sugar lower in deficient rats than normal ones. Stress (anoxic anoxia) resulted in an increase of liver glycogen and blood glucose in normal rats; this response was not obtained in the deficient animals (41). Deb *et al.* (40) reported that deficient female rats had decreased urinary 17-ketosteroids and observed lymphocytosis and eosinophilia indicating adrenocortical hypofunction; however, adrenocortical hormones associated with salt metabolism apparently were not affected since blood sodium and urinary sodium and potassium levels were not altered. Adrenal cholesterol is depressed in pantothenic deficiency (40, 41, 42). However, other factors appear to be concerned with the levels of adrenal cholesterol; thus, extreme seasonal variability of adrenal cholesterol (and ascorbic acid) has been observed (41) in both normal and deficient rats. A great deal of variability in cholesterol levels and its biosynthesis, depending on specific conditions, has also been observed in other tissues (81, 82, 83). Dumm *et al.* (42) examined the effects of various diets on adrenal cholesterol after unilateral adrenalectomy (moderate stress) and after combined adrenalectomy and skin denudation (severe stress). After stress the restoration of adrenal cholesterol was slower in rats on a diet high in ascorbic acid than in those on a normal diet (1 mg. of pantothenate per 100 g. of diet), but it was not affected by a high intake of pantothenate (43.6 mg. pantothenate per 100 gm. of diet). Twenty-four hours after stress, adrenal cholesterol was lower on a diet containing 16 per cent protein than on one with 22 per cent protein. The more severe stress led to a more prolonged depression of adrenal cholesterol than the more moderate unilateral adrenalectomy. Restoration of cholesterol was slower in female than in male rats. The observed adrenocortical hypofunction is probably not the result of pituitary insufficiency in pantothenic deficiency since Butler & Morgan (43) found that the quantities of ACTH in the pituitary and in

venous blood of pantothenate-deficient rats was the same as that in paired fed controls.

Hazelwood *et al.* (44) examined the effect of growth hormone on urinary nitrogen excretion of rats on control and pantothenic acid-deficient diets. Nitrogen retention was greater on the control diets. Similar effects with growth hormone on the two diets were obtained after adrenalectomy. Ringler, Becker & Nelson (45) found that the CoA content of guinea pig mammary tissue rose to a high level following parturition; however, under hormonal treatment sufficient to develop the gland and initiate milk secretion, these changes were not evident (46).

Ginuchi *et al.* (47, 48), in confirmation of the findings of Ludovici *et al.* (49), observed that pantothenic acid-deficient rats had lowered antibody formation. A reduced capacity to form hemagglutinins to human erythrocytes and agglutinins to *Salmonella typhosa* and *Brucella melitensis* was found on the pantothenate-deficient diets; however, these effects could be counteracted by the addition to the diet of 0.05 per cent chloramphenicol (chloromycetin), chlortetracycline (aureomycin), penicillin, and dihydrostreptomycin (48). The decreased level of antibodies is presumably also responsible for the greater susceptibility to *Corynebacterium* infection of young rats on a diet low in pantothenic acid (50). Zucker *et al.* (50) also reported that adult rats fed such a ration had duodenal mucosal lesions and that, regardless of age, there was an increased incidence of pediculosis in pantothenic-deficient rats.

Maas (51) has continued the studies of pantothenic acid synthesis in extracts of *Escherichia coli*. In previous investigations (52, 53) this series of reactions was formulated in the following manner:

$$\text{Enzyme (E)} + \text{ATP} \rightleftharpoons \text{E-PP} + \text{AMP} \qquad 1.$$

$$\text{E-PP} + \text{pantoate} \rightleftharpoons \text{E-pantoate} + \text{PP} \qquad 2.$$

$$\text{E-pantoate} + \beta\text{-alanine} \rightarrow \text{pantothenate} + \text{E} \qquad 3.$$

This scheme was consistent with the observation that pyrophosphate (PP) and adenylic acid (AMP) were formed in stoichiometric amounts during the course of the reaction and that PP^{32} can exchange label with ATP in the presence of pantoate. However, since label from C^{14}-AMP is not incorporated into ATP under any conditions it is unlikely that free AMP is involved in a reversible step in the sequence of pantothenic acid synthesis. In order to accommodate these findings the following scheme has been suggested for the sequence of reactions leading to pantothenate formation (51):

$$\text{E} + \text{ATP} + \text{pantoate} \rightleftharpoons \text{E-AMP-pantoate} + \text{PP} \qquad 4.$$

$$\text{E-AMP-pantoate} + \beta\text{-alanine} \rightarrow \text{pantothenate} + \text{AMP} \qquad 5$$

In the presence of ATP, pantoate, extract, and hydroxylamine, pantoylhydroxamic acid and PP are formed in stoichiometric amounts though only at 0.02 the rate of the over-all reaction (51). The formation of pantoyl-AMP (or E-AMP-pantoate) is analogous to the demonstration by Berg

(54) that acetyl-AMP and PP can lead to the formation of ATP in the presence of acetate activating enzyme, which is suggestive of the following mechanism (54):

$$\text{ATP} + \text{acetate} \leftrightharpoons \text{acetyl-AMP} + \text{PP} \qquad 6.$$

$$\text{Acetyl-AMP} + \text{CoA} \leftrightharpoons \text{acetyl-CoA} + \text{AMP} \qquad 7.$$

In the case of the pantothenate system evidence was obtained for the formation of pantoyl-AMP, since transfer of O^{18} from the carboxyl group of pantoate to AMP could be demonstrated in the whole system. The formulation of the reaction sequence involving an E-AMP-pantoate complex, rather than one involving "free" pantoyl-AMP, has been suggested since the addition of inorganic pyrophosphatase does not lead to a displacement of equilibrium away from ATP plus pantoate in the absence of β-alanine (55).[5]

A system capable of catalyzing the synthesis of pantothenate from pantoic acid and β-alanine was also found in extracts of *Brucella abortus* strain 19 (56). ATP was required for the reaction. The affinity of this enzyme preparation for β-alanine ($K_s = 4 \times 10^{-5} M$) was much greater than for pantoic acid ($K_s = 5 \times 10^{-2} M$). A point of difference between the results with *Brucella* extracts (56) and those from *E. coli* (57) appears to be in the position of equilibrium between the precursors and pantothenic acid. With the *Brucella* extracts only 0.2 to 0.3 per cent of the substrates was consistently converted to pantothenic acid at pH 7.4 regardless of increased concentrations of ATP, pantoate, β-alanine, or enzyme in contrast to reports of 99 per cent conversion of substrates to pantothenate in *E. coli*. In the experiments with *Brucella* the progress of the reaction was followed by measurement of pantothenic acid production with *L. arabinosus* 17-5. Since this organism does not respond well to pantothenic acid conjugates (64), it is conceivable that if such products were formed they would not be detected; it is also possible that conjugates of pantothenic acid which might form in such extracts might inhibit the coupling mechanism between pantoic acid and β-alanine in *Brucella*.

Pihl & Fritzson (58, cf. 59) have investigated the catabolism of C^{14}-labeled β-alanine in the intact rat. C^{14}-β-alanine labeled separately in each of the three positions was administered. Radioactivity from 1-C^{14}-β-alanine appeared much more rapidly in respiratory carbon dioxide than that from 3-C^{14}-β-alanine and especially that from 2-C^{14}-β-alanine. This suggests that carbon 1 is lost in an early phase of metabolism of β-alanine. Carbon 1 is incorporated into the acetyl group of urinary acetyl-*p*-aminobenzoic acid only to a minor extent, while there is an efficient utilization of carbon atoms 2 and 3. On the basis of the different rates of C^{14} elimination found when 2- and 3-C^{14}-β-alanine were given Pihl & Fritzson favor a pathway of degradation of β-alanine via formylacetic acid→acetaldehyde→acetic acid. Stadtman (60) has obtained conversion of acrylyl-CoA to β-alanyl-CoA with an enzyme purified from an extract of *Clostridium propionicum*.

[5] Maas, W. K. (Personal communication).

Acetobacter suboxydans utilizes all of the compounds postulated as intermediates in CoA biosynthesis more effectively than free pantothenic acid (61, 62). Each of these conjugates is a substituted amide of pantothenic acid, and it is conceivable that other combinations with the carboxyl group of pantothenic acid would also be more active. Several pantothenic acid derivatives, substituted at the carboxyl group, have been prepared and tested with *A. suboxydans* (63, 64). Compounds like methyl pantothenate and pantothenamide were more effective than pantothenic acid though less active than intermediates on the path of CoA synthesis, but other substances, e.g. pantothenyl-β-alanine, pantothenyl-D,L-alanine were less effective. Brown *et al.* (64) found that cell-free extracts could hydrolyze pantethine as well as some of the synthetic derivatives to free pantothenic acid; however, there was no quantitative correlation between the degree of hydrolysis of these substances and their growth-promoting activity for *A. suboxydans.* Pantothenyl-β-alanine and pantothenyl-L-histidine have about the same effect on growth, but, while the former is rapidly attacked, the latter is not detectably hydrolyzed under the conditions employed. It is conceivable that conditions for hydrolysis, e.g., for pantothenyl-L-histidine, are more favorable in the cell or that such a substance might be converted to a more "physiological" form by another mechanism, e.g., transpeptidation with cysteine.

Brown *et al.* (64) observed that the activity of pantothenic acid for *A. suboxydans* was considerably enhanced as the pH of the growth medium was decreased. Variation in pH did not change the response to methyl pantothenate, pantothenamide, or pantetheine. This has been considered to indicate that the cells are more permeable to undissociated pantothenic acid. Variation of pH also influences the activity of pantothenylglycine but not that of pantothenylcysteine; it has been concluded that the presence of a sulfhydryl group promotes the absorption of such substances by this organism. A similar pH effect with pantothenic acid was observed with *L. helveticus* and *Lactobacillus acidophilus* (pantothenic acid was never as active as pantethine for these organisms) but not with *L. arabinosus* and *Lactobacillus casei.* It is apparent from these studies that the effectiveness of a compound in supporting the growth of an organism cannot be taken as a definite indication that the compound is on the direct path of CoA biosynthesis. The same conclusion was reached by Pierpoint *et al.* (65).

Evidence has been presented from studies with enzyme preparations from microorganisms (66) and from rat liver (67) for the following sequence of reactions leading to the formation of CoA from pantothenic acid:

$$\text{pantothenate} \xrightarrow{\text{cysteine}} \text{pantothenyl cysteine} \xrightarrow{-CO_2} \text{pantetheine}$$

$$\xrightarrow{\text{ATP}} \text{4'-phosphopantetheine} \xrightarrow{\text{ATP}} \text{dephospho CoA} \xrightarrow{\text{ATP}} \text{CoA.} \qquad \text{A.}$$

Levinton & Novelli (68) demonstrated a pantetheine kinase in pigeon liver and a similar enzyme was observed in extracts of *L. arabinosus* by

Baddiley *et al.* (69). Ward *et al.* (70) have purified such a kinase from extracts of *Proteus morganii.* The purified enzyme reacts with both pantethine and pantothenic acid. The product from pantothenic acid has been identified as 4′-phosphopantothenic acid. It appears certain from cross inhibition studies that the same enzyme is involved in the phosphorylation of both substrates. In view of the demonstration of a pantothenic acid kinase, a second pathway of CoA formation has been suggested (70):

$$\text{Pantothenate} \xrightarrow{\text{ATP}} \text{4}'\text{-phosphopantothenate} \xrightarrow{\text{cysteine}} \text{4}'\text{-phosphopantothenyl cysteine}$$

$$\xrightarrow{-\text{CO}_2} \text{4}'\text{-phosphopantetheine} \xrightarrow{\text{ATP}} \text{dephospho-CoA} \xrightarrow{\text{ATP}} \text{CoA.} \qquad \text{B.}$$

The natural occurrence of 4′-phosphopantothenic acid, e.g., in spent growth medium of *Neurospora crassa* and cell-free extracts of *A. suboxydans,* has been indicated (64, 70). However, one cannot be certain whether it was formed directly by the action of pantothenic acid kinase or was a product of hydrolysis of phosphopantetheine. It is conceivable that some organisms contain kinases specific for either pantothenic acid or pantetheine; a sharper differentiation between pathways A and B would be possible if this occurred. Such a possibility is suggested by the studies of Wieland *et al.* (71) with C^{14}-pantothenic acid. When pantothenic acid labeled in carbon 3 of the β-alanine portion was added to growing cultures of *Streptobacterium plantarum* 10S, radioactive dephospho-CoA, 4′-phosphopantetheine, pantothenylcysteine, CoA, and pantothenic acid could be identified in the reaction mixture after incubation (see pathway A). In a similar experiment with *Saccharomyces carlsbergensis,* phosphopantothenic acid was also identified. The results with *S. carlsbergensis* have been considered as evidence for pathway B (71). 4′-Phosphopantothenylcysteine would also be expected to be formed if pathway B does occur; however, it was not detected in these studies. Furthermore, the required conversion of 4′-phosphopantothenylcysteine to 4′-phosphopantetheine has not been demonstrated as yet. A sequence of reactions similar to pathway B would provide a partial explanation for the greater relative activity of free pantothenic acid as compared to its conjugates observed with certain organisms (cf. 2). Pantethine and pantetheine were almost as active as pantothenic acid in the chick growth assay (72). However, pantothenylcysteine was inactive for pantothenic deficient chickens or rats (73). The administered compound (injected or dietary) apparently either cannot penetrate to the site of conversion to pantetheine or is not cleaved at a rate adequate for the animals to utilize the pantothenic acid portion. It is of interest in this connection that Pierpoint *et al.* (65) have expressed doubt about the adequacy of the characterization of pantothenylcysteine as an intermediate in CoA synthesis. In any case the detailed enzymatic mechanism of the conjugation of pantothenic acid and cysteine (66, 67) and that of the decarboxylation of pantothenylcysteine have not yet been described. It would be interesting to know if a pantothenyl-

AMP compound [analogous to pantoyl-AMP (51)] is formed as an intermediate of the reaction between pantothenic acid, ATP, and cysteine. The participation of pyridoxal phosphate in the decarboxylation of pantothenylcysteine to pantetheine has been suggested (74); however, it is difficult to reconcile this with the need for a free amino group of the substrate in other B_6-linked amino acid decarboxylases (75). A direct decarboxylation of the cysteine derivative to pantetheine would be the simplest mechanism. However, a sequence of reactions involving oxidation to a pantothenylcysteine sulfinate followed by decarboxylation to the corresponding ethane sulfinic acid [analogous to the reactions of free cysteine in microorganisms and animals (76, 77)], and finally reduction to pantetheine might not be impossible.

Sen & Leopold (78) have described the biosynthetic preparation of P^{32} and S^{35} labeled CoA.

Inositol

Lindberg & McPherson (84) have isolated a C-methyl inositol, laminitol, from the low molecular carbohydrate fraction of the brown alga, *Laminaria cloustoni*. The empirical formula, number of hydroxyl groups, and results of periodate titration indicate that this compound is isomeric with the known C-methyl inositol, mytilitol (C-methylscyllitol[6]), from the mussel *Mytilis edulis* (85, 86) and the synthetic product, isomytilitol (2-C-methyl-*myo*-inositol), which was prepared by Posternak (86). The melting points of laminitol and its hexacetate differ from the corresponding mytilitol and isomytilitol. Furthermore, laminitol migrated at a different rate than mytilitol and isomytilitol on paper chromatograms. Considerations of the greater prevalence of *myo*-inositol compounds in nature and of the low value of the optical rotation of this naturally occurring C-methyl inositol have led the authors to assign a tentative configuration corresponding to 1-C-methyl-*myo*-inositol or 4-C-methyl-*myo*-inositol for laminitol. Laminitol was also present in *Fucus spiralis* and *Desmarestia aculeata* (87). The green algae, *Enteromorpha compressa* and *Chlorella*, Strain Tx 14–10, contained *myo*-inositol (88).

Desjobert (89a) obtained an inositol monophosphate by the chemical hydrolysis of phytic acid in a slightly alkaline medium. An inositol monophosphate was also prepared from phytic acid by enzymatic[7] hydrolysis by Fleurent (cf. 90, 93). Fleury *et al.* (90) compared these inositol monophosphates with the synthetic *myo*-inositol-2-phosphate of Iselin (91). The three inositol monophosphates could not be differentiated by melting point, titration curves, rate of acid hydrolysis, or rates of periodate oxidation. However, infrared spectra and x-ray diffraction patterns indicate that inositol monophosphate prepared by enzymatic hydrolysis and synthetic *myo*-inositol-2-phosphate are identical but differ from that prepared by chemical hydrolysis. The fact that the enzymatic hydrolysis of phytic acid leads to the

[6] Nomenclature of Fletcher *et al.* (89).

[7] Enzyme prepared from wheat bran [Fleury, P. (Personal communication)].

formation of *myo*-inositol-2-phosphate indicates the stereochemical specificity of the phosphatase. It seems a remarkable coincidence that the oxidation of *myo*-inositol by *A. suboxydans* also occurs at position 2. Desjobert & Fleurent (92) have reported the isolation of an inositol monophosphate as well as an inositol diphosphate from the hydrolysis of phytate with a partially purified enzyme from wheat germ.

Herken & Maibauer (94, 95, 96) have examined the release of inositol from various tissues by incubating slices from different organs in a buffered medium. Free inositol (as determined by microbiological assay) was liberated into the suspension fluid from brain and sympathetic ganglia but not from liver, pancreas, thyroid, adrenals, and kidney. The release of inositol from nervous tissue occurred at equal rates under aerobic and anaerobic conditions (95) and is presumably attributable to the hydrolysis of the bound forms. On the basis of these experiments and the observation that the inositol content of cerebrospinal fluid is about three times higher than that of the corresponding serum, it has been suggested that brain tissue has an active inositol metabolism (96).

Hartmann & Gerth (97) kept rats on a diet free of fat and low in lipotropic substances. Such animals developed fatty livers; the lipide composition was higher in neutral fat and lower in phosphatides than that of rats on control rations. The dietary administration of inositol to rats on the deficient rations led to a lowering of hepatic neutral fat, an increase of cholesterol and phosphatide fraction. The utilization of P^{32} for phosphatide synthesis, impaired on the deficient diet, was improved by inositol though never quite restored to normal. It has been proposed that inositol stimulates protein catabolism and thereby leads to the liberation of body methionine which is actually responsible for the lipotropic effect (97). Ghiringhelli *et al.* (98) found no protection by inositol against the development of fatty livers in rats on a high-fat low-protein diet. Neither did the administration of inositol affect the rate of liver regeneration of partially hepatectomized rats on either a normal or high-fat low-protein ration; however, there was a marked reduction in mortality in the operated animals receiving inositol.

Szulmajster (99) found that certain haploid yeast strains would grow on a synthetic minimal medium in the presence of uracil if inositol was added. The combination of uracil and inositol could be replaced by cytosine, uridine, or uridylic acid. Other purine or pyrimidine bases, nucleosides, or nucleotides were inactive. Of all polyalcohols, cyclitols, or inositol phosphates tested, only *myo*-inosose-2 could quantitatively replace inositol. Inositol seems to take part in a biochemical process which permits the transformation of uracil, probably to uridine or cytosine.

The effect of phytic acid on calcium and iron absorption has been discussed in two reviews (100, 101).

Campling & Nixon (102) have measured the inositol levels of blood and other fluids by yeast assay in humans, sheep, monkeys, cats, rabbits, and goats. In all species the free inositol concentration of the fetal blood

was consistently higher than the maternal blood of the same species. Curiously, the inositol concentration was found to be higher in the fetal blood of twins than that of singletons in sheep and man. The high inositol concentration of the fetus is not reflected in an increase in inositol in adult blood during pregnancy. In view of the absence of placental transfer or of synthesis by the placenta in sheep it has been suggested that the extra inositol is synthesized by the fetus.

Daughaday *et al.* (103) have presented evidence for the incorporation of glucose carbon into inositol by rats and chick embryos. Uniformly labeled glucose was administered to rats in several doses over a period of three days. Inositol was isolated from the carcass and the livers of these animals. The specific radioactivity of liver inositol was similar to that of glycogen isolated from the same source. There is a remote possibility that the incorporation of carbon from glucose into inositol is accomplished by intestinal bacteria, even though glucose was administered intraperitoneally and the intestinal content was carefully excluded before fractionation. Radioactivity was found in inositol 64 hr. after C^{14}-glucose was applied to the chorioallantoic membranes of chick embryos. Intestinal bacteria were not a factor in these preparations, of course. These experiments indicate that mechanisms exist for the endogenous synthesis of inositol in animal tissues.

Riboflavin

Grainger *et al.* (104) observed a high incidence of hydrocephalus, occular defects, and skeletal abnormalities in offsprings of rats raised on a diet containing soybean protein as the principal nitrogen source and deficient in various known vitamins. The omission of vitamin B_{12} resulted in hydrocephalus, eye defects, and an increased incidence of bone defects, while a deficiency of riboflavin had no effect on the incidence of hydrocephalus or the eye defects, but resulted in increased skeletal abnormalities. Jones & Baumann (105) obtained increased growth of rats with dietary antibiotics under conditions in which thiamine, riboflavin, or pantothenic acid were present in limiting concentrations. Da *et al.* (106) have studied the riboflavin requirement of adult humans on Indian diets by means of the saturation technique. Typical Indian vegetarian and nonvegetarian diets which supplied 0.83 to 1.35 mg. of riboflavin daily were found adequate to meet the minimum requirement for this vitamin. Terrill *et al.* (179) established the requirement of growing pigs at 0.4 to 0.65 mg. per lb. of diet at 53°F.; the role of riboflavin in swine nutrition has been reviewed (107). Irinoda *et al.* (108) have presented some evidence which is suggestive of a relationship between riboflavin and niacin utilization by animals.

Forker & Morgan (109) have examined the effect of stress on liver glycogen in riboflavin deficient and repleted rats. Rats on a diet supplemented with riboflavin showed a ten-fold increase in liver glycogen in response to anoxia; no effect was obtained in animals which had been on a deficient diet for three weeks. However, the administration of adrenal cortical extracts or

cortisone to deficient animals restored the response to anoxia. Since adrenalectomized rats do not respond to anoxia regardless of the status of riboflavin nutrition, the results suggest that riboflavin is concerned in the elaboration of certain adrenal hormones. Guggenheim & Diamant (110) observed that rats maintained on a diet deficient in riboflavin or choline exhibited a delayed diuretic response to water load.

A number of derivatives of riboflavin have been examined for riboflavin activity. DeRitter *et al.* (111) found that synthetic FMN was as active as riboflavin on a molar basis in all tests used. Oguri & Hayashi (112) demonstrated that the recovery of riboflavin deficient-rats proceeded equally well with fed monomethylolriboflavin, tetraacetylriboflavin (cf. 113), or riboflavin-5′-phosphatomonodiethanolamine. High levels of riboflavin have been reported to afford partial protection of rats against the enlargement of spleen, liver, kidney, and lung, but not testis, caused by 4-(dimethylamino)-azobenzene (114).

The biological effects of a series of riboflavin analogues on microorganisms and rats have been examined (45). 6-Chloro-7-methyl-riboflavin (116) was a potent inhibitor of growth for *L. casei.* It could replace riboflavin in the rat growth assay, but administration of the analogue in larger quantities led to an increased incidence of mortality. 7-Chloro-6-methylriboflavin (117) was more inhibitory for *L. casei* than the 6-chloro-7-methyl analogue; however, it had practically no activity in the rat (115). The demonstration of diethylriboflavin monophosphate in the livers of rats receiving diethylriboflavin (115) provides evidence that such an analogue can be converted to a form suitable for attachment to the protein portion of certain flavoproteins. Examination of the effects of phosphorylated riboflavin analogues on various enzymatically active flavoproteins might provide an insight into the mechanism of inhibition of these analogues.

Flavotin (9(1′-D-sorbityl)isoalloxazine) has been found to potentiate the inhibition by 8-azaguanine of the 755 tumor of C-57 mice, although flavotin alone is ineffective (118). Dietrich & Shapiro (119) observed that flavotin antagonized tumor xanthine oxidase *in vivo;* liver xanthine oxidase activity *in vivo* or *in vitro* was not significantly affected. The inhibition of tumor xanthine oxidase by flavotin would lead to an accumulation of xanthine from guanine. Since xanthine was found to inhibit the action of guanase, its accumulation would result in decreased deamination of 8-azaguanine and thereby to a greater effectiveness of the drug. The combination chemotherapy of flavotin and 8-azaguanine led to similar results when tested on other mouse tumors (lymphoma II and mouse mammary carcinoma E0771) (120). The *in vivo* effects of flavotin could not be counteracted by the administration of FMN in these studies (120), while the action of flavotin on the 755 tumors of C-57 mice has been reported to be reversible by FMN (118).

Yagi *et al.* (121) have described procedures for the separation of riboflavin, FAD, and FMN by ion exchange resins. Whitby (122) and Sili-

prandi & Bianchi (123) have described improved procedures for the preparation of FAD. Gaudiano & Cingolani (126) and Cerletti & Siliprandi (125) separated flavin derivatives by paper electrophoresis. The chemical synthesis of FAD was accomplished by Christie *et al.* (124).

A large number of foods have been analyzed for riboflavin content by the lumiflavin and microbiological (*L. casei*) method. The results of the microbiological and chemical methods were in agreement (127). An average total flavin of 1.16 μg. per gm. of human aorta was found; the flavin level tended to decrease with age (128). A normal flavin content of 25 to 33 μg. per gm. wet weight of human liver was reported. A level considerably lower than normal was found in some cases of liver cancer (129). Lust *et al.* (130) analyzed maternal and cord blood at term for riboflavin, FMN, and FAD. Yagi (131, 135) studied the distribution of flavin derivatives in various tissues of rats after the subcutaneous injection of flavins. The injection of riboflavin resulted in a marked rise of riboflavin in the intestinal mucosa with a moderate increase of FMN, but no change in FAD. The urinary excretion of riboflavin has been reported to be higher in severely alloxan diabetic rats than in normals (132). A slight reduction of blood riboflavin levels of rabbits suffering from acute poisoning from thallium carbonate has been reported (133). Comparisons of the spectrophotometric, polarographic, and fluorometric methods for the determination of riboflavin in pharmaceutical preparations were made (134).

Theorell & Nygaard (136) investigated the dissociation of the "old yellow enzyme" (OYE) and its resynthesis from FMN and apoenzyme[3] by means of a fluorescence recorder. The advantage of this method is its greater sensitivity and ability to cover a much greater range of concentrations than the spectrophotometric procedure. The fluorescence of FMN is quenched when it has reacted with the apoenzyme to form the holoenzyme. The kinetics of the recombination reaction were found to be of the second order and the average magnitude of the constant K_1 was $10.2 \times 10^{-4} M^{-1}$ sec.$^{-1}$ In view of the knowledge that the combination of FMN and protein involves the formation of at least two bonds (137) the lack of variation of K_1 with time and with different degrees of saturation of the protein indicates that the attachment of the first linkage of FMN to apoenzyme favors the combination of the second to such an extent that the course of the second order reaction is not affected and that half-coupled intermediates are not detectable. A value for K_2 of 13.4×10^{-4} sec^{-1} was obtained. The reaction was dependent on OYE concentration and followed first order kinetics. The effects of pH, anions, and temperature on the equilibrium of OYE was also investigated (138). It was found that FMN rapidly associates with the protein only in the pH range where FMN is in the doubly anionic form. The authors consider the protein to be akin to an anion exchange with the

[3] $\text{FMN} + \text{apoenzyme} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{OYE}$

weakly positively charged groups located in a spatial arrangement favorable to the attachment of the doubly negative phosphate group of FMN. Evidence has been presented (139) that these positive binding sites on the protein are primary amino groups, since low concentrations of formaldehyde decreased the association velocity at pH values above neutrality and, in the presence of 0.4 *M* sodium chloride, enhanced the rate of dissociation of OYE under alkaline conditions. Acetic anhydride and phenylisocyanate also inhibited K_1 and decreased the combining capacity of FMN with apoenzyme. Anions of strong acids and polyvalent anions enhanced K_2 and decreased K_1, presumably by displacement of FMN from the binding sites on the protein. There was practically no effect of formaldehyde on the association of riboflavin with apoprotein, indicating that the primary amino groups of the protein bind the phosphate group of FMN. The combination of the isoalloxazine portion of riboflavin and FMN might occur via a hydrogen bond between the imino group and a tyrosyl group of the protein. Thus

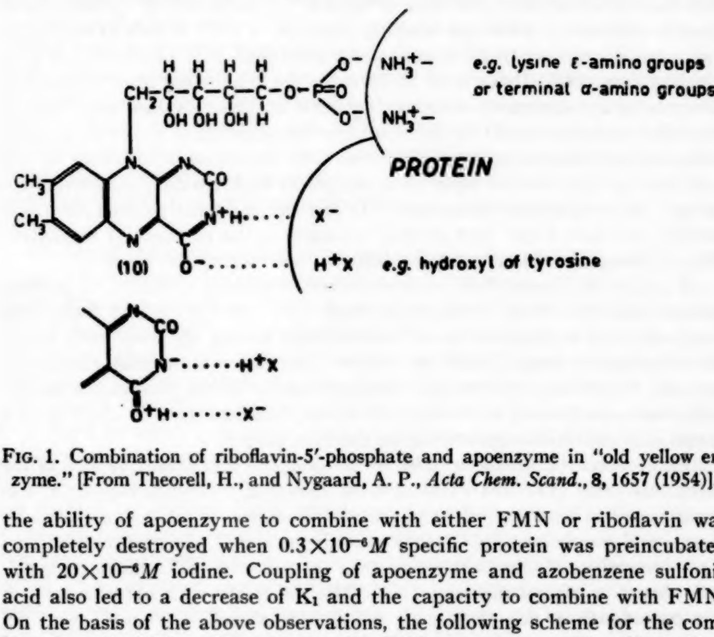

FIG. 1. Combination of riboflavin-5′-phosphate and apoenzyme in "old yellow enzyme." [From Theorell, H., and Nygaard, A. P., *Acta Chem. Scand.*, **8**, 1657 (1954)].

the ability of apoenzyme to combine with either FMN or riboflavin was completely destroyed when $0.3\times10^{-6}M$ specific protein was preincubated with $20\times10^{-6}M$ iodine. Coupling of apoenzyme and azobenzene sulfonic acid also led to a decrease of K_1 and the capacity to combine with FMN. On the basis of the above observations, the following scheme for the combination of FMN and apoenzyme in OYE was presented by Theorell & Nygaard (138) (see Figure 1).

In contrast to OYE the fluorescence of the flavin is not quenched when the apoenzyme of purified D-amino acid oxidase from pig kidney is coupled to FAD (140). The experiments indicate that in the case of D-amino acid

oxidase the phosphoric ester group and the adenine portion of the FAD molecule are involved in the binding to the apoprotein.

Avis *et al.* (141, 142) have obtained milk xanthine oxidase in crystalline form. The main crystalline fraction had a Q_{O_2} of 2300 at 23.5°C. A protein to flavin ratio of 5.0 to 5.2 was found, and the ratio for the cofactors was FAD:Fe:Mo::2.0:8.1:1.4 per unt of protein (cf. 143). Highly purified preparations of xanthine oxidase have also been obtained from calf liver by Kielley (144) and from chicken liver by Remy *et al.* (148). The purified oxidase from calf liver had a ratio of FAD:Mo:Fe: of 1:1:4; that from chicken liver 1:1:8. In the presence of 2,6-dichlorophenolindophenol the calf liver enzyme catalyzed the oxidation of xanthine, DPNH, and acetaldehyde at relative rates of 100:48:24. The carbonyl compounds and DPN appear to operate at different sites on the enzyme, since acetaldehyde interferred with xanthine oxidation but DPNH did not. This is in agreement with observations on the milk enzyme (145) and with inhibitor studies (149). Xanthine oxidase from calf liver catalyzed the reduction of cytochrome-*c*, ferricyanide, and 2,4-dinitrophenol by xanthine or DPNH only at extremely low rates, in contrast to the enzyme from milk (145, 146, 147) and that from chicken liver (148). Doisy *et al.* (149) made the interesting observation that the rate of cytochrome-*c* reduction by milk xanthine oxidase could be enhanced five- to sevenfold by the simultaneous addition of a chelating agent such as 8-hydroxyquinoline and an iron salt. Oxidation of xanthine by the calf liver enzyme was as rapid with oxygen as with 2,6-dichlorophenolindophenol while oxidation by oxygen with the enzyme from chicken liver proceeded at a rate 1 per cent of that obtained in the presence of methylene blue. These purified enzymes also differ in their absorption spectra.

Nicholas & Nason (150) have examined the mode of action of purified nitrate reductase from *Neurospora*. Both FAD (or FMN) and molybdate were reported to function as electron carriers during the enzymatic transfer of electrons from TPNH to nitrate. The observation that chemically reduced molybdate could reduce nitrate to nitrite in the presence of nitrate reductase would seem to be the first direct demonstration of the role of a metal in a metallo-flavoprotein as an electron carrier.

Flavoproteins are known to be involved in the first oxidative step of the fatty acid cycle (157, 158). One of these enzymes, a cuproflavoprotein, was highly purified from beef liver particles and shown to be homogeneous by Green and co-workers (151). This enzyme catalyzed the oxidation of acyl CoA derivatives of short chain length in the presence of 2,6-dichlorophenolindophenol or of cytochrome-*c* (152). The reactivity with cytochrome-*c* or ferricyanide (but not that with 2,6-dichlorophenolindophenol) was decreased when the metal was removed by dialysis against cyanide solution, but could be restored upon the addition of copper salts to the dialyzed preparation (152). The rate of reaction of this enzyme with cytochrome-*c* can be enhanced by the addition of certain quinones (153). Crane *et al.* (154, 155) have reported the preparation of three fatty-acyl-CoA dehydro-

genases from pig liver. Two of these are yellow flavoproteins (Y_1 and Y_2), while the third is a green cuproflavoprotein (G). Y_1 has a broad range of substrate specificity while Y_2 does not react at a significant rate with butyryl-CoA. G appears to be akin to the green enzyme from beef liver (151). Reaction of these flavoproteins with suitably acyl-CoA derivatives led to the reduction of the flavin portion which was followed spectrophotometrically. However, in contrast to the enzyme from beef liver (151, 152) these flavoproteins cannot be reoxidized by 2,6-dichlorophenolindophenol, ferricyanide, or cytochrome-*c*. Another flavoprotein (electron transferring flavoprotein, ETF) is required for the interaction of these dehydrogenases with the artificial electron acceptors. Reduced ETF does not react rapidly with cytochrome-*c*, but when this preparation is purified further two fractions can be obtained, one of which now reacts rapidly with cytochrome-*c* while the other acquires DPN and TPN cytochrome reductase activity. Upon recombination of the two fractions, activity of cytochrome-*c* disappears again, both with ETF and reduced pyridine nucleotides. These findings appear to complicate the explanation of the role of metals in catalytically active metalloflavoproteins as a bridge for the intramolecular channeling of electrons from a two-step (flavin) to a one-step (e.g., cytochrome-*c*) oxido-reduction process (156, 159, 160). The requirement of the acyl-CoA dehydrogenases from pig liver for additional factor or factors to react with certain electron acceptors raises the possibility of similar linking enzymes being found upon further purification of other catalytically active flavoproteins.

Studies of the incorporation of labeled organic compounds into riboflavin by growing cultures of *Ashbya gossypii* revealed a structural as well as precursor relationship between rings B and C of this vitamin and the purines (161) and, more recently, the pterines (162). Klungsøyr (163) has confirmed the observation that C^{14}-formate is incorporated into carbon 2 of riboflavin using *Eremothecium ashbyii*. He also found the carboxyl carbon of acetate incorporated into this position (164), in contrast to the experiments with *A. gossypii* in which acetate-1-C^{14} contributed to carbon 4 (162). Klungsøyr proposed that the carboxyl group of acetate is converted to formate by *E. ashbyii*. The conversion of part of the purine structure into riboflavin was studied by McNutt (165), who found that the extent of incorporation of radioactivity from adenine-8-C^{14} into riboflavin was much less than that from randomly labeled adenine. This might be expected if the purine is converted to a precursor compound common to riboflavin and the purines; carbon 8 would be lost and an *o*-xylene portion would be substituted. A likely intermediate in such an interconversion is 4,5-diaminouracil; however, Brown, Goodwin, & Pendlington (166) found that this compound did not stimulate the production of riboflavin by *E. ashbyii*. Goodwin *et al.* (166,167) and McNutt (165) confirmed the observation of McLaren (168) that certain purines stimulate the formation of riboflavin by *E. ashbyii*, in the light of the studies by McNutt, it is curious that 2-methyladenine was quite an

effective flaviogenic agent (166). The distribution of label in ring A from C^{14}-glucose and acetate has been investigated with *A. gosspyii,* the pattern suggests that the aromatic ring is derived from two-carbon precursor compounds (169, 170). Goodwin & Pendlington (167) observed stimulation of riboflavin production by threonine, proposing the direct structural contribution of the amino acid to the aromatic ring. The incorporation of C^{14} from acetate and glucose, labeled in various positions, into the ribityl portion was examined (171, 172). The label pattern suggests that both the transketolase and the hexosemonophosphate oxidative pathway are involved in the formation of the riboflavin side chain from glucose. It is apparent from these studies with growing organisms (172) that the label distribution in the ribityl group [as well as in the aromatic ring (169)] depends upon the nature of the carbohydrate metabolism predominating in a particular microorganism, even though the ultimate precursor compounds and their enzymatic assembly to form riboflavin may be quite similar in all cases. The solution of this problem can probably be obtained only by studies with enzyme systems and the identification of more direct precursors on the biosynthetic pathway of the vitamin.

Smiley & Stone (173) observed that the inclusion of 0.005 to 0.03 *M* propionic acid in a grain stillage or corn steep liquor containing medium enhanced riboflavin production with *A. gossypii.* Riboflavin synthesis was enhanced by the addition of 2,2′-bipyridine to potato starch mashes for *Clostridium acetobutylicum* (174), while Mitra (175) found an enhancement of flavin yields with BY2 yeast upon the addition of iron salts. Tirunarayanan *et al.* (176, 177) observed inhibition of riboflavin production by *Aspergillus oryzae* with γ-hexachlorocyclohexane, γ-(3,4-ureylenecyclohexyl) butyric acid, or biotin sulfone; biotin could overcome the inhibition. The cytology of *E. ashbyii* has been investigated with particular regard to the distribution of riboflavin in this organism (178).

Thiamine

Hoff-Jørgensen & Hansen have reported a microbiological assay for thiamine using the yeast, *Kloeckera brevis* B768 which can determine as little as 0.001 μg per ml. with a standard deviation of less than 10 per cent. Growth of the organism is not supported by the two major fission products of thiamine, 2-methyl-4-amino-5-aminomethylpyrimidine dichloride or 4-methyl-5-(β-hydroxyethyl) thiazole. Cocarboxylase has 30 per cent less growth promoting activity than thiamine but is fully as active after mylase treatment (180). The fungus, *Tuberculina persicina,* elaborates a pigment into the medium when thiamine is added to growing cultures (181). The quantity of pigment as measured spectrophotometrically is proportional to the quantity of thiamine added, and this has been suggested as the basis of a thiamine assay. Kline (182) has recommended the deletion of the Association of Official Agricultural Chemists' fermentation method for thiamine determination since the chemical determination by the thiochrome method has proved its dependability.

Several publications concerned with the thiamine requirements of microorganisms, cells in tissue culture, and insect larvae have appeared. Eagle (183) demonstrated that thiamine, in addition to several other water-soluble vitamins, was required for the survival and multiplication of mouse fibroblast and human carcinoma cells in tissue culture. The optimum thiamine concentration was 10^{-8}gm. per ml. Fothergill & Ashcroft found that thiamine was required for the growth of *Venturia inaequalis*, an ascomycete which causes apple scab (184). The plant parasite, *Labyrinthula spp.*, which is a marine organism, also showed a requirement for thiamine (185). The addition of thiamine to cultures of *Tetrahymena pyriformis* (Y) [also called *Tetrahymena geleii* (Y)] retards the onset of senescence (186). Larvae of the blowfly, *Phormia regina* (Meig), require thiamine, in addition to other water-soluble vitamins, for growth (187). Steinman *et al.* (188) studied the growth factor requirements of a saprophytic treponeme (S-69) which required cocarboxylase. For optimum growth 10 $\mu\mu$g. per ml. was necessary. Thiamine monophosphate was less than 0.1 per cent as active while thiamine was weakly active at a level of 0.1 mg. per ml.

Harris (189) by studying the growth requirements of various strains of *Neurospora* concluded that there are two alternate pathways of thiamine biosynthesis in this organism. The predominant pathway involves the condensation of the pyrimidine moiety with a precursor of the thiazole moiety to form a thiamine-like intermediate which is then converted to thiamine.

Germ-free and normal chicks exhibited similar requirements for thiamine, though the deficiency was somewhat more acute in germ-free animals (190). The excreta of deficient chicks contained appreciable quantities of vitamins, the presence of which cannot be attributed to microbiological synthesis in germ-free birds. Anderson & Parker (191) found that there was no interaction between manganese and thiamine when incorporated into the diet of rats. Earlier studies had indicated that thiamine deficiency was aggravated by increasing the manganese content of the ration. The minimal thiamine requirement for optimal growth of the baby pig has been determined to be 1.5 mg. per kg. of dietary solids intake with a synthetic diet containing 10 per cent fat (192).

Studying tissue thiamine levels and varying the fat content of the ration, Gershoff & Hegsted (193) stated that the rate of thiamine loss was dependent on the thiamine content of the tissues and was essentially independent of the level of fat in the diet. Likewise, thyroxine failed to modify the rate of thiamine loss. Holt & Synderman (194) suggested that the tissues studied may not reflect the rate of thiamine loss from the body. They found that with human infants the substitution of carbohydrate for fat resulted in a diminished urinary excretion of thiamine. Thiamine excretion could be brought up to the level obtained with the higher fat diet by increasing the dietary thiamine intake. Therefore, they see no reason to question the concept of the thiamine sparing action of fat.

Various metabolic aspects of thiamine deficiency have been studied. Confirming his previous observation (195), Salem has demonstrated by

means of paper chromatography that methylglyoxal appears in the urine of thiamine-deficient rats (196). Monfoort (197) found that in deficient pigeons the pyruvic decarboxylase and α-ketoglutaric decarboxylase of breast and heart muscle declined as the deficiency progressed. The total decrease of the pyruvic decarboxylase was greater than that of the α-ketoglutaric decarboxylase activity. The apoenzymes were completely maintained during most of the 51 day deficiency period and were only partially lost during the last stages of the deficiency. The adenosine-5′-phosphatase activity of whole chicken brain was slightly decreased during thiamine deficiency. However, by histochemical studies of the cerebral hemispheres of normal animals, it was found that the fibers of the main bundles and the nuclei were stained with about equal intensity, while in deficient animals the fibers did not stain and the nuclei showed increased activity [Naidoo & Pratt (198)]. The thiamine pyrophosphatase and alkaline phosphatase, but not the acid phosphatase of the optic tectum, were increased in the deficient chick (199). The thiamine phosphatase of chick brain is distinct from that of rat brain. It is activated only by Mg^{++} and exhibits optimal activity at pH 8.4.

When rats were kept on a thiamine-deficient diet for 30 days the hypophysis was reduced in size with attendent changes in the cellular distribution of the gland. The changes were believed to be a result of the effects of the deficiency on the nervous system (200). Stimulation of the sympathetic autonomic center in the hypothalamus of the rabbit caused a considerable decrease in the thiamine content of liver and kidney and a slight increase in lung, heart, spleen, and adrenal. Stimulation of the parasympathetic center had no effect on the thiamine level of these organs (201).

Rindi and co-workers have continued their studies of the relationship of adrenal function to blood pyruvate level in thiamine deficiency (202). In rats the rise in blood pyruvate level appears to be more closely correlated with adrenal hypertrophy than with the decrease in tissue thiamine. In adrenalectomized or hypophysectomized deficient animals the rise is not as marked as in deficient controls (203, 204); similarly the administration of cortisone was found to increase the level of blood pyruvate (205). The authors suggest that the hyperpyruvicaemia which accompanies thiamine deficiency is a result of two factors: (*a*), the stress situation which is nonspecific and (*b*), the slower process of thiamine depletion.

The injection of cocarboxylase into normal subjects caused an increase in the urinary excretion of 17-ketosteroids, 11-deoxycorticoids, and particularly of 11-hydroxycorticoids (206). The importance of this relationship would depend on whether or not it is merely a nonspecific stress response. In patients who succumbed to heart failure the thiamine and cocarboxylase level in heart muscle, liver, and kidney was decreased as compared to controls who died as a result of other causes. This might be attributed to the previous dietary history of the patients (207). In the light of the other studies cited the prolonged stress accompanying the disease, in the presence of undernutrition, may also be a factor which bears consideration. In studies

of the relationship of thiamine to reproduction in rats Nelson & Evans (208) expressed the belief that the decreased food intake in thiamine deficiency is an important factor in the reproductive upsets encountered, and that in the deficiency state there is an inhibition of the production of maternal sex hormones as evidenced by the maintenance of pregnancy by the administration of estrone and progesterone.

Dalgliesh has been interested in the relationship between thiamine and tryptophan metabolism (209, 210). When tryptophan was fed to young rats on a thiamine and pyridoxine-deficient diet, the animals initially exhibited the typical excretory pattern of pyridoxine deficiency. As the thiamine deficiency progressed, the excretion of kynurenine, hydroxykynurenine, and the N-acetyl derivatives, and xanthurenic acid decreased. Eventually only hydroxykynurenine was excreted. If thiamine was then administered, there was a marked growth response, and the typical pyridoxine deficiency excretory pattern was re-established. Tryptophan feeding in thiamine deficiency alone did not elicit the typical excretory pattern, and there also was no evidence of formylkynurenine excretion. It was concluded that thiamine is involved in the conversion of tryptophan into formylkynurenine.

Thiamine has been implicated in the nitrogen metabolism of germinating seeds of *Phaseolus radiatus* (211). Addition of neopyrithiamine results in an accumulation of glutamic acid and a decreased synthesis of aspartic acid and asparagine. The effect of neopyrithiamine can be partially reversed by thiamine and almost completely reversed by cocarboxylase. The authors suggest that the inhibition may be at the ketoglutarate step of the Krebs cycle with a resulting diminution of the synthesis of oxalacetate and asparagine. They also noted that neopyrithiamine treated rats did not tolerate the intraperitoneal administration of glutamic acid. This was taken as further evidence for the existence of the above mechanism. Another study implicates thiamine in nitrogen metabolism. Survival time of thiamine-deficient rats injected with pyrithiamine daily was decreased as the protein level of the diet was increased. This was not the case with the animals which were deficient, but not injected with the antagonist. Apparently a severe deficiency must be obtained before this protein effect can be elicited in the rat (212).

Toxopyrimidine phosphate, the analogous phosphate of the pyrimidine moiety of thiamine, was synthesized by treating 2-methyl-6-amino-5-hydroxymethylpyrimidine (toxopyrimidine) with phosphorus oxychloride. Toxopyrimidine phosphate was found to be a competitive antagonist of pyridoxal phosphate in the tyrosine decarboxylase system of *Streptococcus faecalis* (213).

Two publications suggest interesting but unexplained relationships between thiamine and vitamin B_{12}. Saxena and co-workers (214) found that the addition of 0.01 to 1.0 μg. of vitamin B_{12} to cultures of wild strain *E. coli* resulted in a 20 to 55 per cent reduction of thiamine synthesis. Employing *E. coli* 113–3 for the assay of vitamin B_{12} in multivitamin preparations, low values were obtained as compared with those obtained with the *Lactobacillus*

leichmannii assay [McLaughlan *et al.* (215)]. This was attributed to the thiamine present in the preparations. Prior extraction of the samples with sodium metabisulfite destroyed the thiamine and permitted use of the organism for vitamin B_{12} assay. Thiamine has also been reported to be capable of destroying vitamin B_{12} in heated aqueous solutions at pH 4.0 (216).

A suspension of resting cells of *Saccharomyces cerevisiae* in phosphate or acetate buffer took up thiamine only in the presence of glucose after a lag phase of 15 to 20 min. [Ziro (217)]. This occurred under aerobic or anaerobic conditions and continued until all the glucose was utilized. Cells which had been preincubated with glucose took up thiamine aerobically in the absence of added glucose. To a lesser extent the same was true for ethanol and acetic acid. Thiamine uptake was inhibited by NaF, iodoacetate, NaN_3, As^{3+}, and 2,4-dinitrophenol except in cells which had been preincubated with glucose.

Ogata *et al.* (218) studied the incorporation of phosphorus into the terminal P of ATP from P^{32}-cocarboxylase. The transphosphorylation proceeds rapidly in liver homogenates or in a washed residue preparation supplemented with Mg^{++}, K^{+}, P*i*, and ATP in the presence of α-ketoglutarate or pyruvate. Succinate is ineffective in this regard. The rate of incorporation from cocarboxylase is far greater than from Pi^{32} and is proportional to the cocarboxylase concentration. The incorporation is inhibited by NaF but not by 2,4-dinitrophenol. Tissues of thiamine-deficient rats showed diminished incorporating ability.

The addition of O,S-diacetylthiamine to a medium containing segments of rat small intestine resulted in contraction of the segments. This effect was prevented by pretreatment of the tissue with atropine. Simultaneous addition of choline and O,S-diacetylthiamine gave stronger contractions than with the thiamine derivative alone. On the other hand, thiamine suppressed the contraction of loops treated with acetylcholine, while O,S-diacetylthiamine and thiamine alkyl disulfide had no effect. The addition of O,S-dibenzoylthiamine caused relaxation of the intestine and a decrease in the response to acetylcholine. It appears from this study that acetylcholine can be synthesized from O,S-diacetylthiamine and choline in rat intestine (219). De (220) has found that thiamine is an inhibitor of human serum cholinesterase. Complete inhibition was obtained at a level of $7.5\times10^{-3}M$, and could not be reversed by the addition of acetylcholine.

Cerecedo has recently reviewed thiamine antagonists (221). Oxyneopyrithiamine (2-methyl-3-β-hydroxyethyl-N-[(2-methyl-4-hydroxypyrimidyl-5-methyl)] pyridinium bromide) has been synthesized and its activity compared with that of neopyrithiamine [Cerecedo & Eich (222)]. This new compound, the 4-hydroxypyrimidyl derivative of neopyrithiamine is ineffective as an inhibitor of the ATP-thiamine phosphokinase system of rat liver. In growth studies with mice it was also ineffective as a thiamine inhibitor. Neopyrithiamine was an effective antagonist in both cases. This study demonstrates the necessity of the 4-aminopyrimidyl group in neo-

pyrithiamine for its inhibitory properties. The same authors (223) studied the effects of neopyrithiamine, oxythiamine, and of oxythiamine diphosphate on the decarboxylation of α-ketobutyric acid by purified wheat germ carboxylase. Only oxythiamine diphosphate inhibited the decarboxylation. Similar results were obtained earlier with pyruvate as substrate (224). From studies employing thiamine antagonists in mice Wooley & Merrifield (225) postulate that thiamine has two important functions in animals. One is its incorporation into cocarboxylase. This is inhibited by oxythiamine and not by pyrithiamine. Still somewhat obscure is the need of thiamine for proper functioning of the nervous system as demonstrated by pyrithiamine antagonism, but not by oxythiamine. It was observed that in thiamine deficiency caused by neopyrithiamine, blood pyruvate and liver cocarboxylase levels were normal while oxythiamine raised the blood pyruvate level and depressed tissue thiamine. Naber *et al.* (226) also suggest two functions for thiamine. Chicks hatching from eggs treated with neopyrithiamine developed polyneuritis, while those from oxythiamine treated eggs failed to show neuritic symptoms. In the young chick the growth inhibition ratio was 1/4 for thiamine/neopyrithiamine, and 1/200 for thiamine/oxythiamine. Neopyrithiamine caused polyneuritis while oxythiamine caused some local and generalized tissue edema. Thus both of the groups cited have evidence for the suggestion that the antagonists attack different systems in the body tissues. The mechanisms responsible for these differences would be of considerable interest.

Flavonoids responsible for the thiamine destroying activity of ferns have been isolated and studied with regard to structural requirements for inactivation (227, 228, 229).

Lipoic Acid (Thioctic Acid)

Some studies involving lipoic acid have been covered in other sections of this review. A comprehensive review of the function of lipoic acid is available (230). Reed & Niu have reported an improved synthesis of DL-α-lipoic acid and have reviewed earlier work on the synthesis of this compound (231).

The first demonstration of a growth promoting effect of lipoic acid in rats and chicks has been published by DeBusk & Williams (232). They employed a purified ration containing casein, sucrose, gelatin, minerals, and vitamins in apparently adequate quantities. The addition of 10 to 1000 μg. of DL-α-lipoic acid per kg. of ration increased the growth and food utilization of both rats and chicks; 10,000 μg. per kg. increased chick growth further.

The role of lipoic acid has been the subject of continued study. Dolin (233), using vacuum dried lipoic acid deficient cells of *S. faecalis* 10Cl found that the oxidation of diacetyl with ferricyanide as electron acceptor is a lipoic acid dependent reaction. Diacetyl oxidation by cell-free extracts

was completely dependent upon the addition of cocarboxylase. The extracts contain bound lipoic acid. A scheme has been presented to illustrate the role of lipoic acid in the oxidative metabolism of active aldehyde formed by cleavage of the α-dicarbonyl linkage. The oxidation of α-ketobutyrate by cell-free extracts of the same organism is also lipoic acid activated [Leach, Yasunobu & Reed (234)]. Preliminary incubation of the extract with lipoic acid was necessary for maximum α-ketobutyrate dismutation activity. Isotope studies indicated that the lipoic acid was being bound and that Mg^{++} and orthophosphate were required for binding. Fractionation of crude extracts with protamine sulfate yielded two fractions both of which were required for activity. When extracts preincubated with lipoic acid were similarly fractionated, the precipitate contained the activity. An active system could be reconstituted by incubating the soluble fraction (which was inactive) with lipoic acid and the protamine precipitate from untreated extract.

Seaman (235, 236), employing alumina to remove lipoic acid, studied the acetate-activating reaction in extracts of pigeon liver acetone powder. The removal of lipoic acid resulted in a loss of activity as measured by the hydroxamic acid method. The activity could be restored by the addition of lipoic acid. Studies with the lipoic acid analogue, 8-methyl lipoic acid, have been taken as further evidence for the requirement of lipoic acid for activation of acetate (237). By means of ammonium sulfate fractionation of the liver powder extract (235), one fraction was obtained with very little acetate activating activity, while a second fraction retained almost all the activity. Both fractions were necessary in order to permit removal of the lipoic acid by alumina treatment. The first fraction functioned by splitting the protein bound lipoic acid from the enzyme. The liberated lipoic acid was then adsorbed and removed by the alumina. The use of alumina to remove lipoic acid has been employed with extracts of *T. pyriformis* and *S. faecalis* with a resulting loss of pyruvate dismutation activity. In purified pyruvic oxidase from pigeon breast muscle and in α-ketoglutaric oxidase from pig heart, the lipoic acid is firmly bound and the addition of the lipoic acid splitting fraction was necessary before alumina treatment could be effective. Enzymatic activity was restored by the addition of lipoic acid to the alumina treated preparations (238, 239).

Calvin and co-workers have continued their investigation of the role of lipoic acid in the photosynthetic mechanism. It was observed that the addition of lipoic acid increased the quantum efficiency of the Hill reaction in *Scenedesmus*. It appears that lipoic acid must be metabolized aerobically before this stimulation of the Hill reaction can be observed (240). Aerobic incubation (in the dark) of *Scenedesmus* cells with S^{35} labeled lipoic acid, followed by extraction and chromatography of the extracts, yielded seven radioactive components. The majority of the activity was found in three components, two of which were identified as lipoic acid and lipoic acid sulfoxide. In long term experiments the major radioactive component obtained

was the unidentified third component. Its properties indicate that it may be a lipide-lipoic acid conjugate (241).

Unidentified Factors

In a continuation of studies aimed at the elucidation of the structure of peptides with strepogenin activity Merrifield & Wooley (242) have isolated a pure pentapeptide from beef insulin digests and identified it as serylhistidylleucylvalylglutamic acid. It has an activity of 80 units per mg. as assayed by the *L. casei* procedure (Standard liver extract activity, 1 unit per mg.). An impure heptapeptide, serylhistidylleucylvalylglutamylalanylleucine, with an activity of 100 units per mg. has also been isolated. These peptides represent a very small portion of the strepogenin activity obtainable from insulin. Other components with greater activity have previously been obtained, but their structures have not yet been elucidated (243). A number of synthetic peptides related to oxytocin have been tested for strepogenin activity and an attempt made to determine the structural requirements for an active material [Wooley *et al.* (244)]. Developing sea-urchin eggs have been found to contain two growth factors for *L. casei* which may be related to strepogenin (245).

Gyorgy & Rose (246) studying the nutritional requirements of strains of *L. bifidus* var. pennsylvanicus found that the organism requires in addition to the "bifidus factor" and pantethine, a factor present in pancreatin, milk, whey, casein, and casein hydrolyzed enzymatically or mildly by acid. The factor is a peptide or protein and may be related to strepogenin. The requirement for the additional factor varies with the chemical nature of the "bifidus factor" present in the medium. If N-acetyl-D-glucosamine and alkyl N-acetyl-β-D-glucosamides are used, the requirement for the supplementary factor is greater than when crude charcoal eluates prepared from natural sources are employed to supply "bifidus factor." This interaction must be considered when assaying for the "bifidus factor." Related to this is Huhtanen's finding that pantethine (synthetic *Lactobacillus bulgaricus* factor) and enzymatically hydrolyzed casein could replace crude natural materials (e.g., fish solubles) as sources of an unidentified growth factor for rumen bacterial and avian *L. bifidus* (1). It has been reported that optimal growth can be obtained with *L. bifidus* grown on a completely synthetic medium to which 1 to 2.5 per cent ascorbic acid has been added (247). This is unexpected since Gyorgy (248) incorporates ascorbic acid into media used for assay of "bifidus factor," though not at as high a level as suggested above. It may be that the strain of *L. bifidus* employed is a mutant which no longer requires a source of "bifidus factor." This apparent incongruity requires further investigation.

A new factor for *Lactobacillus casei* present in corn steep has been reported (249). It stimulates growth and the rate of anaerobic dissimilation of glucose. The factor is not of bacterial origin since it is also present in whole corn. Fur-

ther work is necessary in order to establish whether this factor is related to some of the previously described growth factors. The growth of *L. casei* can also be stimulated by the addition of D-α-hydroxy fatty acids (C_8-C_{14}). The corresponding hydroxy acids of L configuration are inactive (250).

Hemin is an *in vitro* growth factor for some strains of *Mycobacterium tuberculosis*. The addition of hemin at a concentration of 0.0001 *M* resulted in a definite increase in growth (251). With isoniazid resistant strains it has been found that hemin must be present in order for isoniazid to exert its inhibitory action (252). The egg yolk growth factor for tubercle bacilli which is present in the nonsaponifiable, ether fraction has been identified as cholesterol. Another factor is present in the residue obtained after extraction with organic solvents (253).

Many publications have appeared concerned with the requirement by the chick of unidentified dietary factors. The variation in the diets used and the state of depletion of the chicks employed in various experiments makes it extremely difficult to compare data from different laboratories. In the last review Johnson has categorized (74) the various chick factors. Several workers have attempted to standardize diets and to study other factors which affect the response of chicks to unidentified nutrients (254, 255, 256). Peterson, Wiese & Pappenhagen found that chicks from hens maintained on deep litter and a ration containing herring meal responded equally to supplementation of a purified assay diet with herring meal, sardine fish solubles, dried whey, Biopar C, or a defatted liver residue. Combining two or more of the supplements did not result in increased growth. This indicated that either only one factor was required or that each supplement contained all the factors required under the conditions of the assay procedure. Another possibility which was considered is that the chicks may not have been depleted of one or more of the unidentified factors. The supplements used would have been expected to supply more than one unidentified growth factor (254). March *et al.* present further evidence that the factors in green feed and herring meal are distinct (257). Schaefer *et al.* (258) have substantiated earlier observations indicating that penicillin and streptomycin mycelium meals contained unidentified factors for chicks on a practical ration. The feeding of calf thymus produced a small additional growth response in the presence of fish solubles, distillers solubles, grass juice concentrate, or dried brewers yeast (259).

The presence of inorganic growth factors has been reported in fish meal (260) and distillers solubles (261). The ash of both substances appreciably stimulated growth of chicks; however, the unashed supplements were more effective and seemed to contain additional organic factors.

In further studies of factors required by turkey poults it was found that dried whey, whey products, dried brewers yeast, and condensed fish solubles had growth promoting activity (262). The data indicate that the factor in whey may be associated with protein and that a common factor may be present in brewers yeast and fish solubles. Dehydrated alfalfa meal, condensed

fish solubles, and distillers solubles supplementation of an all vegetable protein diet for turkey hens increased the hatchability of eggs (263).

A factor which enhances the storage of vitamin A in chick livers has been reported to be present in condensed fish solubles. The factor was very stable to various types of chemical treatment (264).

As with unidentified chick factors, the state of depletion of the animals must be considered in studying the requirement of unidentified factors by the rat (265). Rats may require flavonoid compounds under conditions of stress (266).

Swine on a purified diet do not grow at a more rapid rate when their diet is supplemented with dried brewers yeast, dried whey, whey fermentation solubles, or Menhaden fish solubles. Grass juice concentrate had slight growth promoting activity (267). On a practical ration containing alfalfa a growth response was obtained when digested cod liver mycelium was added. Condensed tuna fish solubles had a variable effect (268). The inclusion of alfalfa into a purified or practical ration fed to sows significantly increased the number of young which were weaned (269, 270). Oxytetracycline and streptomycin fermentation products do not appear to stimulate growth of young dairy calves (271).

Kinetin, a substance which promotes cell division in plants, has been isolated, characterized, and synthesized (272, 273). When tobacco wound callus tissue is grown on agar medium containing indoleacetic acid it will undergo cell enlargement, but cell division will not occur unless a source of kinetin is added. Kinetin activity was present in aged or autoclaved preparations of herring sperm deoxyribonucleic acid, but not in fresh preparations. The active crystalline material was obtained by butanol extraction of the autoclaved deoxyribonucleic acid, followed by chromatography on Dowex 50. It is active at a level of 0.01 p.p.m. The product was characterized as 6-furfurylaminopurine, and the structure was proved by synthesis. The biological activity and physical properties of the synthetic and isolated kinetin were identical.

Intrinsic factor from hog stomach has been highly purified. When assayed in pernicious anemia patients in relapse, the purified material was effective at a level of 1 to 2 mg. daily (274).

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FAT-SOLUBLE VITAMINS[1,2]

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The biochemical function of fat-soluble vitamins at a molecular level is still unknown, but significant advances have been made this year which promise well for the future. A possible systemic effect of vitamin A on polycyclic compounds of sterolic nature; synthesis and metabolic studies of C^{14}-labelled carotene, vitamin A, and vitamin D; a new scheme of photochemical formation of vitamin D; the possible involvement of vitamin E in the respiratory chain and of vitamin K in photosynthetic processes; and the relationship of essential fatty acids with cholesterol metabolism are a few samples of the progress made.

VITAMIN A

A number of reviews have appeared, discussing the mode of action of vitamin A [Morton (1)], its biochemistry, hypervitaminosis, and requirements (2, 3, 4).

Carotenoids have been considered only so far as they relate to vitamin A metabolism, and the interesting developments on biosynthesis of carotenoids have been left for future reviewers of this particular field.

Chemistry.[3]—A full account of the synthesis of various isomers of vitamin A, referred to in detail in last year's review, has been published [Robeson *et al.* (5 to 8)]. Beutel *et al.* (9) have been able to transform vitamin A acetate to *retro*-vitamin A acetate in good yield by treatment with aqueous hydrobromic acid. The resulting red-yellow oil had the typical absorption spectrum of *trans-retro*-vitamin A acetate. In this connection Henbest *et al.* (10) have shown that anhydrovitamin A_2, obtained from vitamin A_2 by treatment with ethanolic hydrochloric acid, is really 4′-ethoxyanhydrovitamin A_1, the latter being a hydrocarbon with a "*retro*"-structure. A suitable method of converting vitamin A_1 into the rarer vitamin A_2 has been described, via conversion of retinene_1 to retinene_2 by treatment with N-bromosuccinimide followed by dehydrobromination (11).

[1] The survey of the literature pertaining to this review was completed in November, 1955.

[2] The following abbreviations are used in this chapter: ADP for adenosine-5′-diphosphate; AMP for adenosine-5′-monophosphate; ATP for adenosine-5′-triphosphate; BAL for 2,3-dimercaptopropanol; DNA for deoxyribonucleic acid; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); FMN for flavin mononucleotide; RNA for ribonucleic acid.

[3] In the numbering system for vitamin A the terminal alcoholic carbon of the side chain is assigned number 1; the term *retro* is used for a change in the conjugated system one carbon back towards the ring, see Quaife, M. L., *Ann. Rev. Biochem.*, **23**, 215 (1954). For the numbering system of carotenoids, see Goodwin, T. W., *Ann. Rev. Biochem.*, **24**, 497 (1955).

A valuable addition to the tools available for studying the pathway of the vitamin, is the synthesis of the radioactive vitamin A-2-C^{14} by Wolf *et al.* (12). A useful procedure of purification of milligram quantities of vitamin A from tissue extracts has been described [Powell *et al.* (13)]. It involves removal of sterols by digitonin and chromatography on aluminum oxide columns; the recovery is reported to be 68 to 89 per cent. Cormier (14) reports studies on the conversion of vitamin A alcohol into vitamin A epoxide.

The important syntheses in the carotenoid series by Inhoffen and collaborators (15, 16, 17) have been continued. They include the synthesis of 10,10′-*cis*-bixinmethylester (15) and the total synthesis of 3,4-3′,4′-bisdehydro-β-carotene (16). The latter was obtained by condensation of a newly prepared *retro*-dehydro-C_{19}-aldehyde to a C_{40}-diol by treatment with acetylene-dimagnesiumbromide. The diol was converted to 3,4-15,15′-3′,4′-trisdehydro-β-carotene which after hydrogenation and isomerization gave the crystalline "all"-*trans*-3,4-3′,4′-bisdehydro-β-carotene. This synthetic pathway has been utilized to obtain a centrally C^{14}-labelled (15,15′)-β-carotene (17).

Analysis.—The precision of the Carr-Price test for the estimation of vitamin A in margarine has been markedly improved by chromatographic purification of the unsaponifiable fraction on a column of defatted bone meal of appropriate particle size [Lord & Bradley (18)]. Bro-Rasmussen and associates (19) describe a satisfactory method for the separate estimation of neovitamin A, all-*trans* vitamin A_1, and vitamin A_2 in fish liver oils. The compounds were separated by chromatography on dicalcium phosphate columns with a mixture of light petroleum. Morton & Bro-Rasmussen (20) have compared the above procedure with that used in Liverpool which employs conversion factors to give aggregate vitamin A activity for mixtures of the three compounds. Both methods lead to much the same estimate of total vitamin A_1 potency. Partition chromatography using paraffin oil as a stationary phase and various aliphatic alcohols as the mobile phase have been used to separate vitamin A from some of the substances interfering with its spectrophotometric assay (21). Caster & Mickelsen (22) evaluated critically the vitamin A assay in serum. The time relation to intensity of coloured vitamin A-complexes has been studied photometrically (23). The Carr-Price reaction for vitamin A in the presence of carotene has been re-evaluated (24).

Lora-Tamayo & Leal (25) prepared the addition product of β-carotene-*p*-benzoquinone in which the ratio of compounds is 1:5 as expected from the existence of five conjugated double bonds.

Biopotency of vitamin A isomers and related compounds.—Ames, Swanson & Harris (26, 27) have published in detail their studies on the biopotency of five isomers of vitamin A acetate and of vitamin A aldehyde; since a full account of their work had been reported in last year's *Annual Review of Biochemistry* by Boyer (27a) no further comments appear to be needed. Karrer & Eugster (28) compared the biological activity of synthetic *dl*-α-carotene with that of the natural *d*-α-carotene and synthetic β-carotene.

While the *d*-α-carotene had, as expected, half the activity of the synthetic β-carotene, the synthetic *dl*-α-carotene had less than half the activity of the natural *d*-isomer. In fact, after four weeks of dosing, all animals showed deficiency symptoms. The authors suggest that the *l*-form acts as an antagonist of the active natural *d*-form, possibly by interfering with the enzyme system responsible for the conversion of carotenes to vitamin A. Eugster, Trivedi & Karrer (29) synthesized 2,2′-dimethyl-β-carotene which proved to be only about half as active as β-carotene.

Astaxanthin esters isolated from the integument of the shrimp, *Aristeomorphea foliacea*, and freed of carotene and vitamin A, cured the xerophthalmia of rats, but did not enable rats to gain weight [Grangaud *et al.* (30, 31)]. In this connection it may be mentioned that the carotenoid pigment astaxanthin has been found in the feather barbs and vermilion skin of the American flamingo and may be responsible for the striking pink colour of the bird [Fox (32)].

Occurrence and distribution.—Kon and associates (33, 34) have continued their interesting studies on carotenoids and vitamin A which occur in certain marine invertebrates and seem to be an important source of vitamin A for marine vertebrates. In 9 out of 11 species of *Euphausiid* crustaceans, vitamin A was found in high concentration, located principally in the eyes. The only carotenoid, in all species, was astaxanthin or its esters (33). From the eyes of the crustacean, *Meganictiphanes norvegica*, two vitamin A components were isolated by chromatography on alumina; one of the components absorbing maximally at 325 mμ was identified as all-*trans* vitamin A. The other component, having a peak at 311 to 312 mμ had properities consistent with its being a *cis* isomer of vitamin A (34). Wald & Burg (35) identified the vitamin A of the lobster eye as the *cis* isomer, neovitamin Ab, the specific precursor of the visual pigment, rhodopsin. The full papers will be awaited with interest to see whether the *cis* isomers found by the two groups are identical (35a). The *neo-b* isomer is evidently the 7-*cis*-vitamin A (35b). Lambertsen & Braekkan (36) also report the isolation of a new vitamin A_1 isomer from the eyes of the deep-sea prawn, *Pandalus borealis;* they claim that it could be the Δ-3,5-di-*cis*-isomer. The results of Dartnall (37) on the visual pigments of the clawed toad, *Xenopus laevis*, have been reinvestigated by Wald (38). He concludes that the retinal pigment is mainly porphyropsin accompanied by a small amount of rhodopsin.

In the search of intermediates participating in the visual cycle, particularly in the change of rhodopsin to "indicator yellow," Morton's group (39, 40) prepared from retinene and methylamine a crystalline indicator-yellow analogue, which is shown to be a Schiffs' base, retinylidene methylamine (39); its methylammonium ions are stable, but the uncharged compound hydrolyzes to retinene. This behaviour may explain the influence of pH on the stability of "indicator yellow" solutions obtained from eyes (40).

The conversion of all-*trans*-retinene to neoretinene b is catalyzed by an enzyme found in retinas of cattle, retinene isomerase, which is activated by

light [Hubbard (41)]. Rushton & Campbell (42) developed a photocell system which permits direct estimation of rhodopsin in the living human eye.

A number of paper report the content of vitamin A of some Egyptian and Indian fishes (43, 44) and of Indian dairy products (45, 46).

Stability.—The preservation of carotene and vitamin A continues to be investigated. Booth published an excellent account of the problem of preventing loss of carotene during storage of dried green crops (47).

The average loss of carotene in chick mash containing 5 per cent of dried grass was, after 16 weeks, 13.5 per cent, while dried grass alone lost only 8 per cent. With dried grass of good quality, the stored chick mash supplied the needs of chicks [Booth *et al.* (48)]. Addition of 5 per cent of animal fats or animal oils to dehydrated alfalfa meal decreased the loss of carotene during storage, despite the content of animal fats of 5 to 40 per cent of free fatty acids [Bickoff *et al.* (49)]. Mitchell & Silker reported that various vegetable or animal oils, applied as antidust agents in concentrations of 0.8 per cent, do not protect carotene in alfalfa meal unless an antioxidant, 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, has been added (50). Similar experiences in mixed feeds are reported by Livingston *et al.* (51). Evidently the protective effect of fats depends on the amount applied and on type of fat used, since fats in some conditions may even accelerate destruction of carotene. The relation between structure of quinoline derivatives and their antioxidant activity has been investigated (52); the 6-alkoxy derivatives were most effective for alfalfa meal, while the 6-hydroxy derivative best protected the carotene in mineral oil (53).

The results of Scandinavian workers on the preservation, formation, and utilization of carotene were summarized in an English edition (54).

The stability of vitamin A was investigated in various mixed feeds (55); in stored poultry diets it was adversely affected by the presence of Fe, Cu, and Co salts (56). Extensive studies were made on the loss of vitamin A potency in Indian foods during preparation and cooking (57 to 61).

The administration in water suspension of preparations of vitamin A palmitate protected with gelatin or pectin (dry vitamin A) is advocated by Bernhard & Heinzelmann (62), but Høie & Sandvik (63) did not find any difference betwen dry preparations of vitamin A and medicinal cod liver oil given to chicks.

Studies of vitamin A deficiency.—An interesting development in vitamin A metabolism is the extensive study of Morton and associates (1, 64 to 67) on the constitutents of the unsaponifiable portion of animal tissue lipides. It appears that a new and abnormal metabolite, a polycyclic compound not derived from vitamin A and designated compound SC, accumulates in vitamin A deficiency (64). It is normally present in small amounts in the liver, kidneys, and intestines of the pig, sheep, dog, and pullets (65) and is considered to be derived from a sterol by dehydrogenation, but its chromophoric grouping has not been identified (1). Less clear is the connection

with vitamin A metabolism of other minor lipide unsaponifiable constituents in liver, namely cholesta-3,5-diene-7-one (66), compound SA [possibly cholesta-8(9)-ene-7,11-dione (66)], and the more polar compound SB, which are also found in small amounts in tissues of normal animals (1). The task of identification is made difficult by the presence of a variety of vitamin A artifacts (65, 68).

The accumulation of the abnormal metabolite is not associated with a marked derangement of the endogenous cholesterol metabolism, since the levels of plasma and liver cholesterol were not changed by vitamin A deficiency [Green, Lowe & Morton (69)]. On the other hand, cholesterol feeding substantially reduced the liver stores of vitamin A in rats and cockerels (70). Migicovsky reports that liver homogenates from vitamin A-deficient rats are unable to synthesize cholesterol from C^{14} -acetate; since starvation produces similar results, it is possible that these effects are nonspecific (71).

Balakhovskii *et al.* report that injection of S^{35}-labelled methionine was followed after 48 hr. by a greater rise of S^{35} in the liver and kidneys of the deficient rats than in the controls; it is suggested that deficiency may inhibit intracellular oxidation and excretion of sulphur (72).

The oxygen consumption of deficient rats was 10 to 25 per cent greater than in the controls (73); the rate of respiration of the isolated diaphragm of deficient rats was also somewhat higher than that of normals (74).

Reduced quantities of deoxyribonucleic and ribonucleic acid were found in a number of organs of young, vitamin A-deficient rats, but not in their kidneys; increased amounts of both were observed in their livers and spleen. Vitamin A therapy restored the DNA and RNA to normal [Niesar (75)].

The dental depigmentation in rats deficient in vitamin A or vitamin E is accompanied by a diminution of the iron content in the depigmented enamel of the incisor teeth [Moore & Mitchell (76)].

Hedenberg investigated the macroscopic and microscopic changes and stone formation in the urinary tract of vitamin A-deficient rats. She finds a localized and marked hyperkeratosis at the base of the bladder, which gives rise to a marked stasis with dilation of the bladder and ureters; bacterial infection and alkaline urine appear to be responsible for the formation of the urinary calculi (77). The epithelium of the oesophagus, stomach, and sometimes of parts of the small intestine of deficient rats shows degenerative changes, but no damage occurs to the epithelium of the tongue or the large intestine [Planel *et al.* (78)].

The development of hydrocephalus with an accompanying stenosis of the cerebral aqueduct has been further studied in foetuses and young animals reared from vitamin A-deficient rabbits (79, 80) and rats (81). The papilloedema and compression of the optic nerves may be a direct result of increased pressure in the subarachnoid spaces in the region of the optic foramina (79). One of the earliest signs of vitamin A deficiency in the chick is an increased pressure of the cerebrospinal fluid (82). Similar findings are reported in

deficient pigs (83). Field cases of blindness in beef cattle were observed, with constriction of the optic nerves, which might be associated with vitamin A deficiency (84, 85).

Effect of excess of vitamin A.—The increased pressure of cerebrospinal fluid in deficiency is difficult to reconcile with the reports that administration to infants of large doses of vitamin A (350,000 I.U.) produces symptoms of transient acute intracranial hypertension, which subside within 48 hr. [Marie *et al.* (86)].

Hedenberg observed that on administration of large doses of vitamin A to rats, the normal epithelium in the vagina and uterus was replaced by a noncornifying epithelium (77), which in appearance simulated the changes obtained *in vitro* with explants from chicken embryos [Fell (87)]. Locally applied vitamin A on the vagina of ovariectomised rats prevented the appearance in the vaginal smear of cornified cells which are a sign of the estrogenic effect of locally applied estradiol. Vitamin A decreased the concentration of protein-bound sulphhydryl groups [Kahn (88)]. Bern *et al.* observed no such reduction in protein-SH groups on local treatment of the male rat epidermis with vitamin A, nor was there any definite evidence of interference with the keratinization process. Vitamin A, locally applied on the male guinea pig epidermis in the nipple region, did not interfere with the hyperkeratotic response to estradiol benzoate. While no definite effects of vitamin A on the stratum corneum could be discerned, the number and size of cells of the stratum germinativum and granulosum greatly increased (acanthosis) after topical application, but not on subcutaneous administration of vitamin A (89).

Excess of vitamin A appears to increase the rate of mitoses in chick heart fibroblasts *in vitro* [Lasnitzki (90)]. Removal of parathyroids in rats had no influence on the changes of bone formation caused by hypervitaminosis A; Cohen *et al.* conclude that the pathological effect of excess vitamin cannot be explained by stimulation of parathyroids. The proteins in the blood of hypervitaminotic rats were reduced and mucoproteins much increased, but the calcium and phosphorus levels were not affected (91). The cholesterol content in rat brain was not changed in hypervitaminosis (92).

Administration of vitamin A (20,000 to 35,000 I.U. daily) to pregnant rats caused in most instances the death and resorption of foetuses. In the survivors there was a high incidence of malformations, which included cleft palate and ocular abnormalities [Giroud & Martinet (93, 94)].

Fully grown foxes and mink tolerate from 200 to 300 I.U. vitamin A per gm. body weight daily for 6 to 8 weeks, but young animals soon show signs of intoxication, such as anorexia, bone changes, loss of fur, exophthalmos, cramp, and local hyperesthesia of the skin [Helgebostad (95)].

Metabolism of carotene and vitamin A.—Instead of the oxidative fission at the central double bond of β-carotene, resulting in the formation of vitamin A aldehyde, which is later reduced to the alcohol, Glover & Redfearn suggest a step-wise oxidative fission from one end of the carotene molecule by a type of

β-oxidation of the α-methyl branched fatty acid chain. Possible intermediates such as β-apo-8′-, β-apo-10′-, and β-apo-12′-carotenals were prepared and found to be converted to vitamin A by rats; β-apo-10′-carotenal underwent β-oxidation to β-apo-12′-carotenol. Vitamin A aldehyde has methyl groups in β-position, hence it would not be oxidized from the open chain but reduced by alcohol dehydrogenase (96). Lycopene undergoes a similar stepwise oxidation, but the resulting lycopenals have no vitamin A activity [Painter & Glover (97)]. The "nibble" theory, as opposed to the fission hypothesis, would explain the fact that one molecule of β-carotene forms only one molecule of vitamin A and that the α- and γ-carotenes have only half the activity of the β-isomer (98).

Fluorescent microscopy has been used to demonstrate the conversion to vitamin A of carotene, given intravenously to vitamin A-deficient rats; vitamin A was detected both in liver cells and to a greater extent in Kupfer cells [Greenberg *et al.* (99)]. Krinsky (100) succeeded in bringing into cell-free preparations the enzyme system of cattle retinas and rat liver that esterifies all-*trans* vitamin A alcohol to its ester. Removal of the supernatant from the particulate fraction of the retinal suspension reduced the esterifying potency; triglycerides, lecithin, fatty acids, ATP, and coenzyme A did not restore the activity.

Laughland found 38 per cent of C^{14} in the expired CO_2 of a chick and similar amounts in a rat, when a single dose of uniformly labeled C^{14}-β-carotene (about 100 μg.) dissolved in cottonseed oil had been administered orally. The peak of $C^{14}O_2$ excretion occurred in the chick 5 hr. after dosing and at 10 hr. in the rat. Surprisingly, only 1 per cent of the administered radioactivity was found in body tissues of the chick, mainly in the liver. The excreta and gut content contained 36 per cent of C^{14} (101). Different results are reported by Krause *et al.* (102) who administered to rats C^{14}-β-carotene dispersed in water; there was apparently no label excreted in the respired CO_2, since the entire administered dose could be accounted for, partly in the nonsaponifiable matter of feces (70 per cent) and the rest in body tissues as nonsaponifiable substances not identical with carotene.

Wolf *et al.* injected intraperitoneally into rats 2.65 mg. of vitamin A-2-C^{14} in a watery emulsion. In the expired CO_2, 5 per cent of the radioactivity appeared; the rate of the appearance of $C^{14}O_2$ over a period of 24 hr. was almost constant. In the excreta 34 per cent C^{14} was found, of which ¾ was in urine in the form of water-soluble, light petroleum-insoluble metabolites. Liver contained 14 per cent of the radioactivity, skin 0.5 per cent, eyes 0.01 per cent, and 24 per cent was found in the remaining carcass (12, 103). Solubilized β-carotene, in Tween dispersion, and injected intravenously into deficient chicks, was rapidly converted to vitamin A which appeared in the liver and serum. Injected cryptoxanthin did not form significant amounts of vitamin A. Rabbits injected with carotene also formed vitamin A which was about equally distributed between the liver and kidneys and which amounted to about 1 to 2 per cent of the dose on equimolar basis [Bieri (104)]. While

Bieri found appreciable amounts of carotenoids circulating in rabbit blood even after 24 hr., Kon *et al.* report that carotene, injected in Tween dispersion, disappeared rapidly from the blood of rabbits and was apparently destroyed. The fast appearance of vitamin A alcohol in blood of rabbits and rats suggests that the intestinal wall was not the site of conversion of the injected carotene. In calves little or no vitamin A was formed from injected carotene, and the circulating carotenoids amounted to only half of the injected dose (105). The conversion of injected carotene to vitamin A was not influenced by administration of up to 20 mg. tocopherol daily [Bieri (106)]. Normal dogs appear to excrete in their urine 14 to 235 I.U. of vitamin A daily (107).

Nutritional studies.—Vavish *et al.* claim that fat-free milk has the property of increasing carotene utilization in rats (108). In human studies, however, no such effect was observed; the fecal excretion of carotene from sweet potatoes remained at a level of 71 per cent; the blood vitamin A and carotene did not increase on supplementation with sweet potatoes together with or without fat-free milk (109).

In dairy cows (110) and in their calves (111) carotene in doses of up to 300 mg. per head did not increase their blood or liver content of vitamin A or carotene.

The plasma vitamin A values of range sheep in Montana varied from 21 to 38 μg. per 100 ml. (112). Merino sheep, given maintenance rations of wheat and wheaten chaff, had low levels of vitamin A in the plasma and showed a high death rate. A single initial massive dose of 500,000 I.U. vitamin A prevented the deficiency (113).

Gillum *et al.* (114) determined the serum vitamin A levels in 514 healthy adults of both sexes over 50 years of age; the mean values were about 60 μg. per 100 ml. irrespective of sex. The serum carotene levels varied widely. Adam & Gutheil (115) estimated vitamin A in infants suffering from various diseases. Serum vitamin A and carotenoid levels were also investigated in chronic hospital patients (116), in liver diseases (117), and in rheumatic fever (118, 119).

One of the clinical aspects of use of vitamin A is its beneficial effect in diseases of the inner ear (120, 121). Tschirren (122) was able to improve with vitamin A therapy the auditory lesions produced in guinea pigs by streptomycin and neomycin intoxication.

Effect of antioxidants and antibiotics.—Addition of 0.01 per cent N,N′-diphenyl-*p*-phenylenediamine to rations appeared to increase the utilization of carotene in cows (123). Chlorinated naphthalenes given to rats or hamsters caused a decrease in liver vitamin A (124) but had no effect on blood vitamin A (125). Octachloronaphthalene decreased markedly the utilization of carotene when given orally to rats, but not when injected (125).

Chlortetracycline (aureomycin) and diamine penicillin increased in chicks the conversion of carotene to vitamin A, as measured by the vitamin content of livers (126). In rats, chlortetracycline increased the response to

vitamin A when tested by the vaginal smear assay in ovariectomized animals (127), but the liver and kidney stores were not affected (128, 129). When carotene was given to rats, chlortetracycline or vitamin B_{12} increased the deposition of vitamin A in liver and kidneys, but penicillin with vitamin B_{12} was ineffective. It is suggested that the two antibiotics have different effects on the microbial synthesis of vitamin B_{12} [High (129)].

The administration of a carcinogen, 3′-methyl-4-dimethylaminoazobenzene, to rats appeared to have the surprising effect of raising the concentration of liver vitamin A [Reiss *et al.* (130)].

Endocrines and vitamin A.—Clark & Colburn (131) report that administration of large doses of cortisone to normal or adrenalectomized rats on stock or vitamin A free diets resulted in a rapid loss of vitamin A from livers and kidneys. Comparison with effects produced by starvation indicate that losses of vitamin A from liver stores during inanition may be mediated through the adrenal gland. Similar conclusions are put forward by Kagan & Kaiser (132) who observed a marked lowering of serum and liver vitamin A of rats subjected to stress by the production of sterile abscesses.

Injections of an anterior pituitary preparation to cows appear to accelerate the mobilization of hepatic reserves of vitamin A into the blood as the alcohol and hence into the milk as both the alcohol and ester (133).

Extending previous studies on hypothyroidism, Arnrich (134) finds that thiouracil-treated dogs absorb and utilize carotene normally but have increased blood levels of vitamin A, carotene, as well as of cholesterol. This finding may be a nonspecific, general effect on lipide levels. In humans no correlation was observed between the plasma levels of vitamin A or carotene and the basal metabolic rate (135). Vitamin A deficiency in rats results in cystic degeneration or atrophy of the parenchyma of the thyroid gland in the centres of the lobes (135a). Danowski *et al.* (136) suggest that administration to rats of large doses of vitamin A (150,000 I.U./kg.) causes alterations in production, release or transportation of the thyroid hormone as indicated by lowering of the circulating protein-bound iodine.

Bo (137) points out that, in contrast to the vaginal cornification [Murray & Campbell (127)], the metaplastic changes occurring in the uteri of vitamin A-deficient rats do not develop when the ovaries have been removed. Contrary to previous conceptions, the squamous metaplasia and keratinization of the uterus is not only influenced by the vitamin A status but depends on the normal functioning of the ovaries.

VITAMIN D

An excellent review on vitamin D has been published [Dam (2)].

Chemistry.[4]—In a series of papers Inhoffen *et al.* (138, 139, 140) and Braude & Wheeler (141, 142) describe the synthesis of model substances rela-

[4] In view of the increasing number of vitamin D isomers the following nomenclature of sterols with an open ring is adopted: *seco* denotes the opened bond; thus vitamin D_2 (III) would be 9,10-*seco*-ergosta-10(19), 5-*cis*, 7-*trans*, 22-*trans*-tetraen-3β-ol.

ted to the conjugated semicyclic triene system of tachysterol. Several *cis* and *trans* isomers of substituted bis-cyclohexenyl-ethylene were prepared and spectroscopically investigated (138, 141, 142). Several new compounds for ring A synthesis were prepared (139) and condensed with Windaus' $C_{21}H_{34}O$-aldehyde or the $C_{19}H_{32}O$-ketone to give triconjugated tachysterol analogues of 9,10-*seco*-ergostatetraene structure, such as the "all"-*trans*-10(5),6,8(14),22-tetraene (VI), "all"-*trans*-1(10),5,7,22-tetraene (VII), and possibly the 6,7-*cis* analogue of tachysterol (140).

Considerably greater difficulties were experienced when the introduction of a methylene group was attempted to form a triconjugated exocyclic triene related to vitamin D. Three laboratories accomplished it this year by application of the remarkable reaction of Wittig & Schöllkopf (143), in which the carbonyl oxygen of a ketone is directly replaced by an alkylidene group; Harrison *et al.* (144, 145) and Inhoffen *et al.* (146) prepared the model triene, *trans*-1-2'-cyclohexylidene-ethylidene-2-methylene-*cyclo*-hexane. Its unsaturated system differs from that of calciferol only in the transconfiguration of the central double bond. Milas *et al.* prepared the 5-methoxy-2-methylene-cyclohexane derivative (147).

An outstanding achievement is the partial synthesis of the *trans*-vitamin D_2, 9,10-*seco*-10(19), 5-*trans*, 7-*cis*, 22-*trans*-ergostatetraene [Inhoffen *et al.* (148)]; this was accomplished by allowing to react with triphenylphosphine-methylidene the C_{27}-ketone obtained through an aldol condensation of *p*-acetoxy-cyclohexanone with Windaus' $C_{21}H_{34}O$-aldehyde. Verloop *et al.* (148a) also report the preparation of *trans*-vitamin D_2 in the form of a crystalline phenylazobenzoate by isomerization of vitamin D_2 in apolar solvents at room temperature in diffuse daylight by the action of iodine. The preparation of new decalins for use in model syntheses in the 9,10-*seco*-D-homo-steroid series is reported [Inhoffen *et al.* (149)].

In a series of papers on ozonolysis, epoxidation, and the infrared spectrum of precalciferol, Velluz and collaborators reinvestigated its possible structure (150, 151); they conclude that precalciferol is a 6,7-*cis* isomer of tachysterol (152) and has not a spirosteroid configuration as previously proposed (150). Independently, Koevoet *et al.* (153a) arrived at the same conclusion. In support of the 6,7-*cis* structure Velluz *et al.* (152) cite also the similarity of the spectrum of a triol, 9,10-dihydroxy, 9,10-dihydro-precalciferol, with the ultraviolet absorption of 10,19-dihydro-vitamin D_2 II obtained by Schubert (153) through partial catalytic dehydrogenation of vitamin D_2 by Raney nickel. Precalciferol would thus be identical with the synthetic 6,7-*cis* isomer of tachysterol of Inhoffen (140).

Velluz *et al.* (154), and Koevoet *et al.* (153a) by the use of small amounts of I_2, were able, in spite of the accepted view of the irreversibility of photochemical changes, to transform precalciferol into tachysterol. Velluz *et al.* also converted precalciferol photochemically into ergosterol and lumisterol with a 3 per cent yield (155).

In view of these findings Velluz *et al.* (152, 155) proposed a new scheme of

photochemical conversion of conjugated dienes into triconjugated 9,10-*seco*-compounds in which precalciferol would be the pivot steroid, so that ergosterol would be converted directly into precalciferol, which would change spontaneously into vitamin D_2; formation of lumisterol$_2$ and tachysterol$_2$ would thus be a side reaction.

Independently, Havinga *et al.* (155a) suggested, from studies with C^{14}-labeled vitamin D_2 and D_3, an essentially similar scheme in which lumisterol and tachysterol are not essential intermediates in the formation of vitamin D; their scheme differs from that of Velluz in certain details which will have to be settled by further experiments; they postulate an unstable intermediate as the pivot steroid.

This new photochemical conversion chain opens interesting possibilities for the *in vivo* conversion of 7-dehydrocholesterol directly into precalciferol$_3$ without an obligatory formation of lumisterol$_3$ and tachysterol$_3$.

Inhoffen *et al.* (156, 157) confirmed the all-*trans* configuration of the crystalline isovitamin D_2 [identical with pyrotachysterol] and isotachysterol; the *u*-tachysterol, 4,6-*trans*, 8(14), 22-tetraene, is also a 6,7-*trans* isomer. Contrary to the suggestion of Braude & Wheeler (141), Inhoffen *et al.* (157) supports, with good evidence, the 6,7-*trans* configuration of tachysterol. The proposal of Sondheimer & Wheeler (158) to assign to vitamin D_2 the configuration of Inhoffen's *trans* vitamin D is not in accord with the results of other authors [Harrison *et al.* (145); Inhoffern *et al.* (148, 156, 157)].

Uniformly labeled C^{14}-vitamin D_2 in the form of the crystalline dinitrobenzoate ester has been prepared by photochemical conversion of C^{14}-ergosterol; the latter was obtained biosynthetically from yeast made, first, sterol deficient and then incubated with acetate-1-C^{14}. Small amounts of C^{14}-lumisterol were also prepared [Kodicek (159)]. Recently, Havinga *et al.* (155a) employed the biosynthetic approach to study the formation of C^{14}-vitamin D_2 from C^{14}-ergosterol in the presence of nonlabeled lumisterol$_2$ and tachysterol$_2$; the C^{14}-vitamin D_2 was isolated as the maleic anhydride adduct.

Milas *et al.* (160) report the preparation and physical properties of four different salts of vitamin D_3 phosphates; the lithium vitaminate, obtained on treatment with phenyllithium, was reacted with phosphorus oxychloride or di-tert-butyl chlorophosphate. Vitamin D_2 was changed by Openauer oxidation irreversibly into a crystalline conjugated ketone, the 9,10-*seco*-4,10(19), 7,22-ergostatetraene-3-one [Trippett (161)]. The 4,4-dimethyl calciferol has been obtained by photochemical conversion of 4,4-dimethyl ergosterol [Cooley *et al.* (162)]. The nature of the complexes of vitamin D_2, as well as of carotene and vitamin A, with γ-globulin was studied by Troitskii & Tarasova (163).

Analysis.—The measurement of radioactivity in the paw of rats given P^{32} for the bioassay of vitamin D has many advantages over the line test (164). The radioactivity of the bone ash of chicks given Ca^{45} has also been used for vitamin assay (165, 166).

Vitamin D_2 can be estimated in pure solution by the formation of adducts with *p*-benzoquinone; the uncondensed quinone is then titrated (167). Ergosterol, tachysterol, and suprasterol II form similar condensation products (168).

Pharmaceutical preparations of vitamin D were estimated spectrophotometrically (169). The vitamin D_3 content of irradiated milk was about 500 I.U. per kg. according to rat and chick bioassays (170).

A critical evaluation of vitamin D assays has been published [Almquist (171)]. Oral doses of vitamins D_2 and D_3 and of tachysterol have a less rapid and less lasting effect on patients with parathyroid insufficiency than when given intravenously (172).

Corbisterol, the 7-dehydrosterol from the bivalve, *Corbicula leana*, had on irradiation about the same potency for rats as irradiated 7-dehydrocholesterol, but had only 1/25 of the potency for the chick [Rosenberg (173)]. Festenstein & Morton (174) studied the distribution of 7-dehydrosteroids in the skin of the pig, in the intestines of ox, pig, horse, and sheep, in herring roe and milt; it is emphasized that the significance of the 5,7-dienes may not only reside in their capacity to act as provitamins.

Relation to Ca and P metabolism.—Engfeldt & Zetterström (175) conclude from biophysical studies on bone tissue of rachitic dogs that in rickets there is not only a disturbance in mineralization but also in the submicroscopic and microscopic organization of the organic matrix; x-ray diffraction studies show that hydroxyapatite crystallites of normal dimensions have an atypical radial orientation in tranverse bone sections, thus following the abnormal radial disposition of collagen bundles in osteoid tissue. Zetterström & Winberg (176) studying the mineral metabolism in primary, vitamin D-refractory rickets, indicate that vitamin D appears to have also a more direct action on bone tissues than the indirect one of increasing the intestinal absorption of calcium and phosphate, since increased mineralization started despite an initially low $Ca \times P$ quotient. Similar conclusions on the basis of rat experiments are put forward by Nicolaysen (177); in support of a concurrent local action on bones, he cites the abnormal histological structure of "calcified" bones of deficient rats injected with calcium salts and their low citric acid content; after vitamin D administration to deficient rats, increased blood calcium levels can be demonstrated which are not caused by increased absorption of the mineral. In contrast Migicovsky & Jamieson (178) emphasize the increased intestinal absorption of calcium as the primary and sole effect of vitamin D, because the vitamin causes an increased Ca^{45} deposition in chick bones when the mineral is given orally but fails to do so when injected. The interpretation of net deposition of Ca^{45} is, however, difficult since it does not take into account the three factors, the sum of which gives the net result; the physical exchange of Ca, true physiological accretion, and resorption of bone salts. Bauer, Carlsson & Lindquist (179) propose an ingenious formula for calculating separately these three factors; they show that vitamin D has a local stimulating influence on bone resorption involving

possibly citric acid metabolism but have no convincing evidence of a local effect on the accretion of bone salts. In a later report Carlsson & Lindquist (179a) cite in support of a local action on bone the indirect evidence that 10 I.U. of vitamin D daily will cause the maximal absorption of Ca^{45} by the intestines; though a one hundredfold increase in vitamin dosage will further increase the blood level of Ca^{45} that is evidently derived from bones. Engfeldt & Zetterström (180), while studying the deposition of P^{32} injected into deficient and vitamin D-treated rats, note a local effect of vitamin D; the rate of uptake of injected P^{32} in the bone fraction, insoluble in saturated ammonium sulfate and apparently derived from true accretion of mineral salts, was markedly increased on administration of vitamin D.

Sobel & Burger (181), in agreement with previous results by Barrett (182), observe that vitamin D increases the level of circulating lead in rats to which lead is being administered. After lead dosing has ceased the vitamin depresses the Pb blood level proportionally to the rise of serum phosphate. This vitamin effect appears to support the intestinal absorption theory as the primary and sole factor in vitamin D action, but lead salts may not be deposited in bones at the same site as the hydroxyapatite crystallites and may thus not be influenced by vitamin D as bone salts are.

The phytic acid in high-calcium cereal diets is not completely hydrolyzed by the intestinal phytase of rachitic rats unless vitamin D is administered. The resulting increased activity of phytase, however, does not liberate enough inorganic P to account for the curative effect of the vitamin; its antirachitic action may be attributable to an improved utilization of inorganic P in the body [Pileggi *et al.* (183)]. Calcium complexed with phosphopeptides from casein is utilized well by chicks, even when vitamin D is not given [Mellander (184)]. Vitamin D causes a slight rise in the Mg absorption of rats [Meintzer & Steenbock (185)].

Relation to citrate metabolism.—The decrease of citric acid content of serum in infants during the first five days of life is not related to the vitamin D intake, Ca/P ratio, or citric acid content of the diet, but there appears to be some correlation between citric acid and Ca levels in serum (186). Citric acid appeared to have an effect on the calcification *in vitro* of epiphyseal cartilage slices of deficient rats only if it was given orally; subcutaneous injection or addition of citrate to calcifying solutions had no beneficial effect. It is possible that increased serum phosphate levels following citrate therapy are responsible for the healing process [Yendt & Howard (187)].

Studies in vitro.—The *in vitro* calcification of rachitic rat cartilage incubated in human serum was decreased when blood of patients suffering from renal insufficiency was used, despite a high $Ca \times P$ product in their serum; the raised Mg blood level may be the inhibitory factor [Yendt *et al.* (188)]. Sections of rachitic human cartilage, demineralized with ethylenediaminetetraacetate, were incubated in $Ca^{45}Cl_2$ solution; the uptake of Ca^{45} was lowered in the young cartilage adjacent to the abnormal hypertrophic region and appeared to be related to the distribution of sulphated mucopoly-

saccharides (189). A calcium-collagen-chondroitin sulfate complex has been implicated in initiating nuclei of crystallization which then may undergo crystal growth to form hydroxyapatite [Sobel (189a, 190)]. A histochemical study of rachitic epiphyseal cartilage during healing has been published (191).

Picard & Cartier (192) claim that cartilage from rachitic rats fixes *in vitro* more P^{32} from inorganic phosphate and hexosemonophosphates than normal tissue but is unable to fix P^{32} of the pyrophosphate groups of ATP; similar results are obtained with intact rats (193). Rachitic cartilage respires only feebly but has an increased glycolytic and glycogenolytic activity (192). Addition of vitamin D_2 or ergosterol in aqueous lecithin causes an inhibition of the anaerobic glycolysis rate of rat liver slices; the inhibition increases with concentration of the steroid for the range of 25×10^{6}–10^{2} μM (194).

The growth-promoting effect of vitamin D on *Lactobacillus casei*, the growth of which was inhibited by linoleic acid or other essential fatty acids, has been further studied (195, 196, 197). The most potent of reversing agents among the sterols tested were vitamin D_2, vitamin D_3, and dihydrotachysterol, all of which have in common a 9,10-*seco*-5,7-diene structure; Ca ions had a similar effect, and the reversing activities were inversely related to the phosphate concentration of the medium. A physico-chemical mechanism affecting the permeability of the cell membrane is proposed, and it is suggested that these findings provide model systems for the study of vitamin D action.

Metabolism of vitamin D.—Vitamin D_2, administered by mouth to deficient rats in doses of 0.25 to 8 mg., was estimated by reversed-phase paper-chromatography in liver, kidneys, and feces; irrespective of the amount given, about 10 per cent of the dose was found in the liver and about 20 per cent in the feces on the first day. The liver vitamin decreased to about half the value on the second day; since the vitamin D content of other tissues did not simultaneously increase, it is thought that liver tissue may be involved in the destruction of vitamin D (198). Uniformly labeled vitamin D_2-C^{14} (159) was given to rats in a single dose of 1 mg.; all the C^{14} was recovered: 70 per cent as breakdown products, mainly in feces and liver, and 30 per cent as vitamin D itself, two-thirds of which was in the feces and the rest in the body. The bulk of the body vitamin was in liver, bones, blood, intestinal wall, and kidneys, in descending order; the occurrence of vitamin D in intestinal wall, bones, and kidneys may be connected with the P and Ca metabolism of these tissues (197).

Since up to 70 per cent of a massive dose of vitamin was converted by rats into inactive breakdown products, it was possible that intestinal microflora might be partially responsible for the destruction. However, feeding of 1 per cent sulfasuxidine had no effect on the recovery of vitamin D in liver and feces [Cruickshank *et al.* (199)]. Experiments with antibiotics also provide no unequivocal answer; chlortetracycline (200) and penicillin (171) in diets appeared to increase the response of rats to small doses of vitamin D, while

Horn & Eder (201) report that penicillin decreased the response in chicks.

In contrast to the relatively large proportion (20 per cent) of the dose of vitamin D recovered in the feces of the rat, the feces of normal subjects and patients with osteomalacia, who received 20 mg. vitamin D_2 by mouth, contained only 0.1 per cent of the dose as biologically active vitamin D (202).

The amount of vitamin D formed on ultraviolet irradiation in the skin and liver of rachitic rats was determined; fur impaired, but did not prevent the effect of ultraviolet light. It is assumed that vitamin D formed in the deeper epithelial layers of the epidermis is directly transferred to other parts of the body (203). Efficient absorption of topically applied vitamin D through the skin of the rat has been confirmed (204).

Other metabolic relationships.—Raiha & Forsander (205) noted that rachitic rats phosphorylated thiamine to cocarboxylase to a lesser degree than did normal controls, and administration of vitamin D restored the cocarboxylase level in blood to normal. It is at present impossible to decide whether this effect is only a nonspecific result of deficiency (205). Any direct participation of the vitamin in enzyme systems presupposes its presence in significant quantities; in heart sarcosomal (mitochondrial) fragments no vitamin D could be detected, and it was concluded that there was less than 0.03 μM vitamin D/mg. sarcosomal protein, which is small compared with the content of other respiratory catalysts (206).

Vitamin D and parathyroids.—Crawford *et al.* (207, 225) noted that in totally aparathyroid rats, a thousandfold increase in vitamin D dosage caused the kidney tubules to reabsorb a smaller percentage of the glomerular filtrate phosphate. This effect was qualitatively similar to that observed previously for parathyroid hormone (206). In intact animals administration of vitamin D may cause a seemingly paradoxical rise in the tubular reabsorption rate; this rise is supposed to be an indirect effect caused by suppression of parathyroid activity (225). Dent *et al.* (208) made the interesting observation that patients with postoperative tetany who had become insensitive to vitamin D_2 therapy responded promptly to doses of vitamin D_3 or dihydrotachysterol.

The difference between vitamin D and dihydrotachysterol has been narrowed down, since the latter has not only a catabolic effect on bone calcium, but can also increase the intestinal absorption and retention of Ca in the body of patients with hypoparathyroidism (208) and of patients with rickets resistant to vitamin D (209). Carlsson & Lindquist (209a) reached similar conclusions from experiments on rats.

With particular interest will be awaited the full account of the short report of Raoul *et al.* (210, 211) that the lipide, nonnitrogenous fraction of parathyroids contains the active Ca-raising principle which appears to be a sterol possibly related to neo-vitamin D_3 (212); to the latter has been recently assigned the remarkable structure of 9,10-*seco*-5-cholestene-3,10-diol-7-one [Raoul *et al.* (212a)].

Effect of excess of vitamin D.—Hypervitaminosis D in rats and rabbits

causes kidney lesions which in the glomeruli resemble those of intercapillary glomerulosclerosis in man; the blood vessels were mostly dilated, with spasms in the efferent arterioles (213). Overdosing with vitamin D provoked hyperchloremic renal acidosis (Butler-Lightwood-Albright syndrome); treatment with adrenocorticotropic hormone improved the condition (214). The symptoms of vitamin D intoxication in infants have been described (215).

Clinical studies.—The aminoaciduria in rachitic children involves the excretion of histidine, threonine, serine, glycine, lysine, and glutamic acid, caused probably by the failure of kidney tubules to reabsorb the amino acids which may normally be absorbed in the form of phosphate esters. However, a similar defect is observed in scurvy (216, 217, 218). Methionine administration greatly increased the aminoaciduria in vitamin D-resistant rickets (219).

A new syndrome of "idiopathic hypercalcemia of infancy with failure to thrive" is ascribed to oversensitivity to vitamin D or chronic overfeeding with vitamin D (1000 I.U. or more daily) (220, 221). Another situation which appears to be caused by oversensitivity to vitamin D develops in some patients with sarcoidosis; the dramatic effect of cortisone on the raised blood Ca levels, increased Ca absorption, hypercalcemic symptoms, and renal function led Dent *et al.* to postulate a direct metabolic antagonism between cortisone and vitamin D (222). However, cortisone treatment of rats with hypervitaminosis D did not ameliorate the weight loss, clinical appearance, histological lesions, or the increased urinary phosphate excretion [Cruickshank & Kodicek (223)]. The beneficial action of cortisone in man may be caused, at least in part, by its effect on the renal tubular reabsorption of phosphate as observed in dogs [Roberts & Pitts (224)]. In support of the suggestion of an interaction between vitamin D and corticosteroids may be cited the observation that patients suffering from vitamin D-resistant osteomalacia excrete in urine two abnormal substances which appear to be Δ^4-3-ketosteroids, not identical with cortisone, cortisol, corticosterone or its 11-deoxy,11-dehydro, 11-deoxy-17α-hydroxy derivatives, progesterone or testosterone (197). An interaction is also implied in the findings of Bazzi & Nicolosi (226) that administration of large doses of vitamin D_2 or vitamin D_3 decrease markedly the urinary excretion of 11-hydroxy corticosteroids.

A newly recognized disease, infantile and adult hypophosphatasia, resembles rickets except that hypercalcemia and low alkaline serum phosphatase are present (227, 228); an abnormal metabolite, phosphoethanolamine, has been found in the plasma and urine of patients with the disease (229, 230). The abnormal phosphate monoester may be the physiological substrate for bone phosphatase, unless other phosphate esters are accumulating in the same manner (231). In this connection, it may be recalled that Picard & Cartier (192) reported a failure by rachitic cartilage to utilize pyrophosphate groupings of ATP, while normal ossifiable embryonic cartilage metabolized ATP readily, but not orthophosphates nor hexose-, mono-, and di-phosphates (232).

Vitamin E

Almost 200 interesting communications given at the 3rd International Congress on Vitamin E in Venice, Italy, added to the difficult task of selecting, within the limitations of space, studies of biochemical interest. Several reviews on vitamin E have appeared (2, 233).

Chemistry, analysis.—Green *et al.* (234) used an effective two-dimensional technique which combined partition and reversed-phase paper-chromatography, to confirm the presence of ϵ-tocopherol (5-methyl-tocol) in wheat products; they discovered in cereal grains a new tocopherol analogue, ζ-tocopherol which is a 5,7-dimethyl-tocol, isomeric with β- and γ-tocopherols. As much as 68 per cent of ζ-tocopherol and 16 per cent of ϵ-tocopherol are present in wheat bran; about equal amounts of each, 30 per cent, are found in barley and rye (234, 235). The remaining possible analogue, the 7-methyl-tocol or η-tocopherol, has now been discovered in rice (235a). A revaluation of biopotencies of tocopherols in food stuffs is clearly needed. The biopotency of DL-α-tocopherol acetate is 75 per cent of that of the natural D-α-tocopherol acetate (233, 235b). According to rat resorption tests, the ζ-analogue is almost as active as α-tocopherol (236); according to liver storage tests, the biopotency of DL-ζ-tocopherol is half that of DL-α-tocopherol (237).

The biological response of animals to vitamin E is adversely influenced by the presence in the diet of "stress factors," such as unsaturated fat, *o*-cresylesters, CCl_4, and sulphonamides which may act as pro-oxidants, promote the production of lipide peroxides in body tissues and thus increase the need for vitamin E (2, 238). Similar experiences are recorded by Miller *et al.* (239) when sodium bisulphite was used in the diet of chicks. In contrast anti-oxidants such as diphenyl-*p*-phenylene diamine (DPPD) have a sparing action on vitamin E, as Singsen *et al.* (240) showed in studies on the prevention of encephalomalacia in chicks; Johnson & Goodyear (241) even claim that DPPD can completely replace vitamin E for the rat. Scott *et al.* (242) report the composition of a basal diet for chicks on which the birds develop a vitamin E deficiency apparently not influenced by the presence of anti-oxidants or pro-oxidants.

The disappearance of degenerating muscles fibres of vitamin E-deficient hamsters was used for bioassay of tocopherols; tocopheroxide had the same antidystrophic activity as α-tocopherol, β-tocopherol had one-half, while α-tocopherylhydroquinone and α-tocopherylquinone about one-third activity [West & Mason (243, 244)]. The average tocopherol levels in human and canine blood (245) and of summer and winter butterfat in Canada (246) were reported.

Metabolism of vitamin E.—No fission of the $C^{14}H_3$ group in 5 position of α-tocopherol occurred, when rats were given labeled α-tocopheryl succinate by interperitoneal or oral route; the urinary excretion of C^{14} amounted to less than 1 per cent, and 40 to 75 per cent C^{14} appeared in the feces [Niedner & Johnson (247)]. In rabbits about two-thirds of an intravenous dose of

C^{14}-α-tocopheryl succinate was excreted in the feces, one-third in the urine; about 50 per cent of the fecal radioactivity was α-tocopherol; the urinary metabolite may be a glucuronic acid-conjugated lactone of 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone which has also been isolated from human urine (248, 249).

An oral dose of ζ- and γ-tocopherols is deposited in the ingested form in livers of rats; apparently no methylation to α-tocopherol occurs (237). Oral or intramuscular, but not cutaneous application of DL-α-tocopherol produces a rise in plasma vitamin E (250).

During the later stages of pregnancy the tocopherol levels in human blood tend to rise simultaneously with the concentration of carotene (251, 252, 253). Human milk has a higher concentration of vitamin E than cow's milk, and a marked increase occurs after oral administration of vitamin E (254). Despite low plasma and milk tocopherol levels, cows whose offsprings died from muscular dystrophy reared normal foster calves (233, 255).

Effects of deficiency.—Hove & Seibold produced a fatal liver necrosis in pigs given a low-protein, vitamin E deficient diet containing cod liver oil (256). No liver necrosis developed but microcytic anemia was seen in chicks fed on a vitamin E deficient, liver-necrogenic diet containing American *Torula;* vitamin E or brewer's yeast cured the symptoms (242).

Increased autolysis *in vitro* of kidney tissue of deficient rats was observed by following the release of free amino nitrogen (257). The proteolytic activity of muscles, but not that of kidneys, liver, and spleen, was increased in vitamin E-deficient rabbits (258).

Methylene blue treatment of vitamin E-deficient rats prevented "brown uterus," kidney lesions, and, partially, paralysis but afforded no protection against resorption gestations or hemolysis by dialuric acid [Moore *et al.* (245, 259)]. However, Markees (259a) and Dam (2) observed a protective effect even in the last mentioned lesions. An excellent tabulation of various symptoms and curative effects of various agents can be found in the two recent reviews (2, 233).

Hemolysis of human blood, caused *in vitro* by oxygen, was reduced by prior administration of vitamin E (260). The degree of hemolysis with hydrogen peroxide was related to the plasma tocopherol content (261); a high proportion of infants fed skimmed cow's milk or suffering from steatorrhea showed an increased hemolysis titre (262). Erythrocytes of vitamin E-deficient rats show a strong hemolysis, without dialuric acid, on incubation at 37°C.; feeding of vitamin E or methylene blue provides partial protection. Deficient chick erythrocytes are resistant even to dialuric acid [Christensen & Dam (262a)].

The histological changes in skeletal muscle of deficient rabbits (244, 263), in heart muscle (264), and in tissues of the rat were examined (265). In deficient chicks muscle degeneration developed without additional dietary fat and could be prevented by tocopherol or cystine but not by methylene blue

or antabuse [Dam *et al.* (266)]. Vitamin E-deficient monkeys showed muscle dystrophy and anemia with leucocytosis (267). A combination of vitamin E and vitamin K deficiencies produced heart muscle lesions in mice and rats [Dessau *et al.* (268)]. Deficient guinea pigs and rabbits, but not rats, show nonatheromatous necrotic and fibrotic lesions in the media of the aorta (269); both species have a marked elevation of plasma cholesterol esters and of free cholesterol in muscles, but not in liver, while rats show increased amounts of esterified cholesterol in their livers [Deuel *et al.* (270)].

The serum of deficient rabbits had a lowered albumin/globulin ratio and considerably increased β-globulin (271), and the dystrophic muscles had an increased content of free amino acids with the exception of glycine which was markedly lowered (272). However, no such decrease of free glycine in deficient rabbit tissues was noticed by Dinning *et al.* (273). Azzone & Aloisi report that dystrophic mysosin shows changes in solubility and lowered viscosity (274). Of the muscle protein fractions, prepared according to Herrmann & Nicholas (274a), in late-lactation paralysis, actomyosin rapidly decreases, the insoluble fraction increases, and the soluble fraction remains constant [Rumery *et al.* (275)]. Electron microscope studies are interpreted to show the appearance of the brown fluorescent pigment in smooth muscles (276) and the disappearance of myosin and depolymerization of actin in striated muscle (277).

Dinning (278) considers the increased incorporation of C^{14}-formate into the nucleic acids of liver and skeletal muscle of deficient rats an indication of an increased turnover rate of nucleic acids in these tissues. However, in further experiments Dinning *et al.* (273) note that in contrast to C^{14}-formate, C^{14}-glycine is incorporated *in vivo* into nucleic acid purines of a number of tissues of deficient rabbits to a lesser extent than in controls; they postulate that the differential effect of deficiency cannot be the result of pool sizes, but that vitamin E may be more specifically concerned with formate metabolism. It would affect the exchange reaction of the 2 position of the purine ring without complete degradation of the molecule. This specific effect on formate should stimulate further research. It may be mentioned that Keilin & Hartree (279) found that formate can undergo coupled peroxidatic oxidation specifically in the presence of catalase; investigations on vitamin E deficient tissues of coupled peroxidatic reactions in general, and of catalase in particular, may give interesting results, thus extending previous work on lipoxidase (280, 281) and on hematin-catalyzed oxidation of fats (282).

The increased creatinuria and the elevated specific activity of urinary C^{14}-creatine, following injection of C^{14}-methyl labeled choline or C^{14}-formate to rats deficient in both vitamin E and B_6, seems to be the result of an inability of the dystrophic muscles to retain formed creatine (283). The loss in creatine phosphate of heart muscle from deficient rabbits, without an accompanying decrease in inorganic phosphates and adenosinepolyphosphates, is interpreted in the same way [Mulder *et al.* (284)].

Despite certain similarities of the symptoms of muscular dystrophy in rabbits suffering from various deficiencies, the acetylcholine content of potassium deficient muscles was elevated, while that in vitamin E- or choline-deficient muscles was lowered [Hove & Herndon (285)].

King *et al.* (286) report on unexplained relationship between vitamin E, ferrous or ferric salts, and trace elements in the diet for producing the paralytic syndrome of mice. Tentori *et al.* (287) note that thyroid intoxication in deficient rats accelerates the appearance of muscular dystrophy. However, no correlation appears to exist between plasma tocopherol levels and the basal metabolic rate of hyperthyrotoxic patients (135a); nor does methylene blue protect rats from the symptoms of thyroxine intoxication, but on the contrary aggravates them (288).

In human muscular dystrophy the urinary excretion of arginine, threonine, valine, leucine, arginine, and taurine was increased, while that of creatinine and phosphate was decreased; Hurley & Williams (289) suggest that a system involving possibly vitamins E, B_{12} and folic acid, the sulfur-containing amino acids, as well as arginine and glycine, may be functioning inadequately.

In vitro studies of the carbohydrate metabolism of alloxan-washed diaphragm of normal and vitamin E-deficient rats are indicative of an antagonism, as in the hemolysis test, between vitamin E and alloxan (290); less clear results have been obtained regarding the interaction of vitamin E and insulin on the *in vitro* glycogen metabolism of rabbit liver and muscles (291). The carbohydrate metabolism of normal and diabetic subjects, rats, and rabbits is reported to be affected (292, 293) and not affected (294) by administration of vitamin E.

Enzyme relationships.—An important contribution has been made by Nason & Lehman (295) who demonstrated with purified particulate and solubilized mitochondrial preparations of skeletal muscle of the rat and other species that α-, β-, γ-tocopherols and tocopheroxide can specifically activate the decreased DPN-cytochrome-*c* reductase activity of an aged preparation, especially after it has been extracted with isooctane. Similar results were obtained for the reduction of cytochrome-*c* by succinate. Tocopherylquinone and its hydroquinone were less effective, while the following were inactive: vitamins D and K_1, tocopherol esters, the tocopherol nucleus (penta-methyl-6-hydroxy chroman), lipoic acid, cystine, and a number of antioxidants.

Although the lipide residue from the isooctane extraction could completely restore the enzyme activity, no vitamin E could be detected in the lipide material. Antimycin A inhibited competitively the activating effect on tocopherol of the solubilized unextracted DPN-cytochrome-*c* reductase. Cytochrome-*c* oxidase was not affected by tocopherol. The Baltimore workers suggest that tocopherol (its free radicle) or a conjugated form or an unknown lipide cofactor are part of the respiratory chain occupying a place between $DPNH_2$, or flavin enzymes, and cytochrome-*c*.

That vitamin E is specifically involved in the respiratory chain is further supported by the interesting finding of Bouman & Slater (296) that heart sarcosomal (mitochondrial) fragments contain vitamin E in stoichiometric amounts comparable to those of the known components of the respiratory chain.

It is interesting to note that the place occupied by "tocopherol" has been also assigned to the BAL- and antimycin A-sensitive "intermediary factor" of Slater; he, however, recently suggested that his factor may be directly involved in the over-all catalytic reaction, although it may not be directly concerned in the acceptance of hydrogen atoms or electrons [Slater (297)]. This may also be true of the "tocopherol." If it were not so, one would have to postulate two alternative chains (*a*) for tissues containing vitamin E and (*b*) for those, like bacteria, which do not appear to contain it, as indeed has been postulated for the intermediary factor (297a); moreover, yeast and *Neurospora*, whose cytochrome-*c* reductases show also a tocopherol dependence (295), do not seem to have, as far as the reviewer is aware, any vitamin E. They may, however, contain substances functionally related [compare Scott *et al.* (242, p. 53)]. An indirect catalyzing effect, without it being in the direct hydrogen pathway, of "tocopherol" or "the isooctane-extractable factor," possibly in the manner suggested by Bücher (298) for action of vitamin E or K in lipide-water interfaces, would remove the need for additional hypotheses. The participation of vitamin E in exchange reactions in interfaces would fit in with the concept of the mode of action of fat-soluble vitamins as interface catalysts in contrast to prosthetins [Kodicek (299)].

In any case the above findings will have to be correlated with the concept of action of metalloflavoproteins in which at present no place is left for lipide constituents such as vitamin E [Mahler (300)]. While vitamin E is thus implicated in the respiratory chain system, the evidence for the involvement of vitamin E in coupled oxidative phosphorylation has still to come; the findings of Nason & Lehman (295) and of Bouman & Slater (296) give only circumstantial support. Previous work is contradictory as has been discussed in previous reviews and particularly by Boyer (301).

Recently, Chernick *et al.* (302) suggest, albeit without direct experimental evidence, that uncoupling of oxidative phosphorylation may be responsible for the biochemical lesion in livers of rats given a necrogenic diet containing vitamin E-free *Torula* yeast; the oxygen uptake of liver slices of these rats declined after 30 min. and could be prevented by prior feeding of tocopherol, cystine, or factor 3 of Schwarz. Weinstock *et al.* (303) found the oxygen uptake of intermediates of the tricarboxylic cycle greatly increased in homogenates of vitamin E-deficient rabbits; replacement of ATP by ADP and AMP eliminated the differences in oxygen consumption between the deficient and control livers by increasing the O_2 uptake of the latter. An increased oxygen consumption of liver, skeletal muscle, and adrenal slices, but not of heart and kidney preparations, of deficient rabbits has been noted

by Rosenkrantz (304); the effect in adrenals was also elicited in other deficiencies and in stress reactions. An interdependence with calcium of the enhanced respiration was noted.

The pathological metabolic pattern in dietary necrotic liver degeneration still remains complex. The findings of Chernick *et al.* have been mentioned already (302); the decline in oxygen consumption of liver tissue of rats prior to the onset of necrosis was accompanied by a progressive failure of ketogenesis, lipogenesis, and oxidation of C^{14}-acetate to $C^{14}O_2$ (305). Olson *et al.* (306) note a greatly depressed rate of incorporation of S^{35}-cystine in liver coenzyme A of rats given necrogenic diets with a low vitamin E and sulphur-containing amino acid content; the incorporation, however, into liver protein was normal and so was the uptake of C^{14}-methyl labeled methionine into the protein and choline of liver tissue. An impairment of the function of coenzyme A is also implicated in the decreased synthesis *in vitro* of long-chain fatty acids from C^{14}-acetate by liver slices of diseased rats [Artom (307)].

Other metabolic relationships.—The interrelationship between vitamin B_6 and vitamin E has been further studied in rats deficient in both vitamins; administration of either vitamin E or vitamin B_6 reduced the elevated excretion of allantoin and creatine [Young *et al.* (308)].

Tocopherol supplementation increased the hatchabiliy of the eggs of turkeys given an all-vegetable protein ration but had no effect on egg production and fertility; the administered vitamin was stored in the egg yolk (309). The incidence and severity of perosis in turkeys receiving various levels of phosphate was reduced by vitamin E (310). The rate of conversion of C^{14}-glycine to uric acid in gouty subjects was beneficially affected by long-term vitamin E therapy (311).

The ineffectiveness of vitamin E on the blood clotting mechanism has been confirmed (312, 313). Vitamin E, ascorbic acid, and methylene blue, acting possibly as antioxidants, interfere with the formation of anti-streptolysin antibodies in guinea pigs (314). Of potentially practical importance is the observation of Mason & Bergel (315) that an infection of *Mycobacterium leprae* can be established in rats and hamsters when the animals become vitamin E deficient. Deficient mice show after six months a lipide depletion of the adrenal cortex, subcapsular adenomas, and large masses of brown degeneration (316). The significance of the decrease of vitamin E content of blood in liver diseases has been discussed (317). Unlike phytol administration, feeding of tocopherol to rats does not result in an decreased incorporation *in vitro* of C^{14}-acetate in fatty acids of liver slices; evidently no splitting of the intact phytol side chain occurs in the body (317a).

Vitamin K

Chemistry and assay.—The adsorptiochromism of derivatives of 1,4-naphthoquinone, when chromatographed on alumina, has been studied (318). Reversed-phase chromatography on silicone-coated paper has been used to differentiate vitamin K_1 and related compounds by utilizing their

fluorescence before and after exposure to ultraviolet light or KOH [Green & Dam (319)]. In a series of papers Yamagishi (320, 321, 322) describes the estimation of vitamin K_3 and its derivatives by Scudi's cysteine-HCl procedure or by colour formation of some derivatives with dimethylaniline.

Metabolic studies.—Dam and associates (323, 324) reported interesting studies on the fate of vitamin K_1 injected in aqueous dispersion into chicks and rats; about 70 per cent of a massive dose was found as vitamin K (estimated colorimetrically) in liver and spleen and to a small extent (1 per cent) in kidneys and lungs, none was found in the bile. Only traces of the vitamin were still in the blood 14 hr. after intravenous injection. Ingestion of an anticoagulant, coumachlor, did not appear to affect the vitamin deposition. After oral or intramuscular administration, only small amounts were found in the organs (323). Partial blockage by India ink of the reticulo-endothelial system decreased in chicks the deposition of injected vitamin K_1 in the liver, with a concurrent increase in the spleen. No vitamin K was found in the placenta, foetus, or newborn young when a massive dose had been injected intravenously to pregnant rats (324). Apparently the action of the microflora on vitamin K metabolism in chicks has been overestimated, since germ-free animals have been found to recover spontaneously from a vitamin K deficiency [Luckey, Pleasants & Reyniers (325)].

The importance of bile for the absorption of vitamins K, A, and possibly D has been demonstrated on growing puppies deprived of bile (326).

Feeding of menadione or its diphosphate to cows did not affect the lactic acid development in their milk; addition of menadione to milk reduced the rate of lactic acid formation but resulted in discoloration and off-flavors (327).

Action in blood coagulation.—The beneficial effect of vitamin K on the blood concentration of factor VII (or proconvertin) in congenital deficiency of the factor, in the newborn, and in patients with obstructive jaundice has been restated (328 to 331). Synthetic vitamin K_1 appears to be more efficient than the water-soluble 2-methyl-1,4-naphthohydroquinone dicalcium phosphate (synkavite) (330, 331, 332). The decreased prothrombin level in thyrotoxicosis was temporarily improved by injection or oral administration of synthetic vitamin K_1 or menadione; the failure in the blood clotting mechanism is caused by the impaired liver function [Gordin & Lamberg (333)]. Frost & Spruth (334) report that the hemorrhagic condition in chickens occurring in the field can be controlled by menadione sodium bisulphite; when a hemorrhagic condition was induced experimentally by addition of 0.1 per cent sulphaquinoxaline to the chick ration, the requirement for vitamin K was greatly enhanced, and the sodium bisulphite derivative was at least four times more efficient than menadione. This observation may explain the failure of menadione treatment as reported by Yacowitz *et al.* (335). From comparison of field cases of hemorrhagic disease in chicks with experimental vitamin K deficiency, Cover *et al.* (336) conclude that the two syndromes are definitely distinct.

Unspecified vitamin B_{12}-antibiotic supplements restored to normal the

blood clotting time of vitamin K deficient chicks (337). The *ante partum* administration of vitamin K significantly decreased the incidence of hemorrhagic disease of the newborn (338). However, an important warning was given by Allison (339) of danger of overdosing and appearance of kernicterus and hemolytic anemia in premature infants when more than 5 mg. synkavite per day was injected. These findings were confirmed in studies on vitamin E-deficient rats; while injections of vitamin K_1 or oral administration of water-soluble vitamin K substitutes were harmless, injections of some of the water-soluble derivatives caused intense hemolysis [Moore & Sharman (340)].

The success of intravenous injection of vitamin K_1 in raising the low prothrombin-proconvertin levels of patients under treatment with anticoagulants has been well substantiated by a number of authors (332, 341 to 346).

Enzyme relationships.—In continuation of their interesting studies, Martius & Nitz-Litzow (347) investigated an additional number of anticoagulants for their ability to uncouple oxidation and phosphorylation; in general the uncoupling effect ran parallel with the anticoagulant potency. Vitamin K derivatives with a 5 and 11 carbon side chain in position 3, but not menadione, increased the oxidative phosphorylation of mitochondrial preparations from vitamin K deficient chicks, the vitamin analogue with the shorter chain being less effective. As to the interpretation of these data with regard to the concept that vitamin K acts as a member of the respiratory chain, the present reviewer concurs with the excellent analysis of the situation as presented by Boyer (301) in last year's Review. Of interest is the finding of Kruse (348) that sarcosomal (mitochrondrial) fragments, the carriers of the nonphosphorylating respiratory chain system, contain little or no vitamin K (less than 0.01 μM/gm. protein). Uehara *et al.* (349) report that menadione and some other derivatives, but not *p*-benzoquinone and catechol, increase markedly the oxidation of hexosemonophosphates in the presence of yeast antolyzate.

In line with the hypothesis of Wessels (350), Arnon *et al.* (350a) suggest that vitamin K is involved in photosynthesis by participating together with ascorbate and FMN in anaerobic photosynthetic phosphorylation, a process considered to be different from oxidative phosphorylation [Arnon (351)]; the circumstantial evidence rests on the presence of vitamin K_1 in chloroplasts (2) and the fact that the P^{32} incorporation by isolated chloroplasts into ATP is increased, on irradiation and in absence of oxygen, when various naphthoquinone derivatives are present. Some of the quinoid compounds have vitamin K activity, but some have the biologically important methyl group in position 2 replaced by a side chain containing an aromatic ring (350).

Of interest are the paramagnetic resonance studies on vitamin K and other quinones by Woernley (352) and Blois (353).

Other metabolic effects.—In an excellent review Mitchell (354) summarizes his laboratory studies and clinical radiotherapeutic trials of some

chemical radiosensitizers, particularly of water-soluble vitamin K derivatives. A negative effect of synkavite on survival of *Escherichia coli* and *Saccharomyces cerevisiae* exposed to x-rays has been reported (355). Synkavite produced morphological anomalies in early chick and amphibian embryos (356) and inhibited the proliferation of mouse fibroblasts (357, 358). The mechanism of the antibiotic action on bacteria of vitamin K derivatives has been studied (359, 360).

Essential Fatty Acids

An excellent account of newer concepts on essential fatty acids has been presented by Deuel & Reiser (361, 362, 362a).

Chemistry and assay.—Weedon and associates report the syntheses of oleic, *cis*-, and *trans*-octadec-11-enoic acids with the use of acetylenic acids in anodic "crossed" coupling reactions (363, 364). The synthesis of α-linolenic acid, starting from the condensation of 1-bromopent-2-yne with 2-prop-2-ynyloxy-tetrahydropyran, has been accomplished (365).

The separation and estimation of a number of unsaturated octadecanoic acids by a standard reversed-phase column has been reported by Crombie *et al.* (366); the elution rate increased with the number of double bonds. The electrophoretic movement of higher fatty acids (367) and their behaviour on reversed-phase paper-chromatography have been studied (368, 369, 370). Hofmann *et al.* (371) developed a quantitative procedure for the estimation of saturated, monoethenoid, and branched chain fatty acids in small samples of bacterial lipides. Kenaston *et al.* (372) compared the various methods for determining fatty acid oxidation produced by ultraviolet irradiation. A modified peracid process for making epoxy compounds from polyenic acid esters has been published (373). Schlenk *et al.* reported studies on protection of autooxidizable lipide substances by formation of adducts with urea, thiourea, deoxycholic acid, α-cyclodextrin, and starch (374, 375). The distribution of mono- to hexaenoic fatty acids in human plasma and blood cells has been determined by spectrographic methods [Evans *et al.* (376)].

Metabolic studies.—In a series of important studies Klenk and his collaborators (377 to 380) separated by counter-current distribution and identified a number of C_{20}- and C_{22}-polyene fatty acids from the glycerophosphatides of human and animal brain (377, 378) and liver (379). The type of the trienoic, tetraenoic, pentaenoic, and hexaenoic acids was in agreement with the hypothesis that all C_{20}- and C_{22}-polyene acids of liver and brain phosphatides possess the di-vinyl-methane structure and that they are predominantly of the linoleic or linolenic acid type and formed from them by addition of 2 carbon units (380). Similar conclusions were reached by Steinberg *et al.* (381) who fed to mice and rats methyl linoleate-1-C^{14}, while Klenk (380) studied the uptake of acetate-1-C^{14} by rats and rat liver slices.

Iongh (382) reports that the C_{22}-polyenic acids from human brain phosphatides are biologically active in protecting rats from fatty acid deficiency, thus providing confirmatory evidence for the "6,9 (term.)-hypothesis" of

Thomasson (383, 384) and indicating that the central nervous system should also be affected by deficiency. The previously reported biological activity of the triple unsaturated stearolic acid, Δ^9-octadecanoic acid, could not be confirmed (385). The importance of configuration for biological activity is again re-emphasized by the finding of Privett, Pusch & Holman (386) that *cis*-9,*trans*-12-linoleate has no curative effect on fat-deficient rats; however, the *trans* double bonds derived from the acid are stored in tissue lipides.

Tove *et al.* (387) confirmed that the unsaturated fatty acids of the dietary fat exert a major effect on the fatty acid composition of the depot fat of the rat, but they claim that the nature of the dietary protein is capable of altering, to some extent, the depot fat composition. Blomstrand (388) confirmed, on feeding linoleic-1-C^{14} acid to rats, that the major part of the absorbed acid could be recovered in the thoracic lymph; no difference in the route of absorption of saturated and unsaturated fatty acid was noticed. From liver deposition studies with C^{14}-glycerol in fat-deficient rats, Gidez (389) concluded that essential fatty acids appear to have a lipotropic effect and influence the normal liver glyceride turnover.

Diets low in fat result in significant decreases in the di- and tetraenoic fatty acids and an increase of trienoic acid in the serum of infants (390) and of dogs (391). Basnayke & Sinclair (392) describe the increased permeability and the effect of vitamin B_6 deficiency in rats suffering from an essential fatty acid deprivation. Hydrogenated peanut oil, coconut oil, or lard had a deleterious effect on growth of deficient rats (393, 394) and of guinea pigs (395). Deuel *et al.* (396) and Privett *et al.* (386) reaffirm that linolenic acid is considerably less effective biologically than linoleic acid, but addition of linoleic to linolenic acid has a "sparking" effect on the latter by increasing its potency above that of linoleic acid. A similar synergistic effect is observed for the protective action of unsaturated acids against x-irradiation (397). Male rats deficient in essential fatty acids showed early symptoms of degeneration of the seminiferous tubules of the spermatogenic tissue (398), particularly when hydrogenated peanut oil had been included in the diet (399). No correlation between the polyenic acid level in serum with parakeratosis in pigs was observed (400).

There is growing evidence that the intake of unsaturated fatty acids influences profoundly the cholesterol level in blood and liver; deficient rats showed an increased cholesterol content in the liver and a decrease in plasma (394). The hypercholesterolemic response to cholesterol feeding in rats on a sulfur-deficient diet was largely prevented by dietary fat containing a high level of essential fatty acids and by supplementation with methionine (401). In chicks addition of 10 per cent peanut oil resulted in a decreased cholesterol content in liver, but feeding of large amounts of cholesterol caused a reversed effect, namely the fat-deficient chicks showed a lower liver cholesterol than the supplemented ones (402). These differences may be partly attributable to the effect of fats on absorption and on the esterase activity of the intestine (403, 404). The most convincing proof of an inter-

relationship between cholesterol and essential fatty acids is provided by the observation of Peifer & Holman (405) that inclusion of 1 per cent cholesterol in the diet precipitated the fat deficiency within two to four weeks, while comparable deficiency symptoms on fat-free diets without cholesterol were observed only after three months. In a preliminary report Hammond & Lundberg (406) observed somewhat increased values of tri- and hexaenoic fatty acids in blood plasma of atherosclerotic subjects; the significance of these findings is partially marred by the fact that few samples were examined and because of the inherent inaccuracy of the method, but the results would point to a metabolic error common with situations when a diet low in essential fatty acids had been given (391, 392).

Enzyme relationships.—Unsaturated, more than saturated fatty acids, increased the level of alkaline phosphatase in the intestinal mucosa (407). The lipoxidase-catalyzed oxidation of polyunsaturated fatty acids differs to a certain extent from the autooxidation of these acids in that the enzymic process produces, apart from hydroperoxides, significant amounts of dimeric products with conjugated diene systems which are optically active [Lundberg (408)]. The hemin- and plant lipoxidase-catalyzed oxidation of linoleate and its inhibition by antioxidants has been further investigated (409, 410). A cortisone-copper complex catalyzed the autooxidation of linoleic acid; other 17-hydroxycorticosteroids were inactive (411). Ultraviolet-irradiated methyl esters of linoleate and linolenate inhibited a number of enzyme systems of rat liver mitochondria (412).

Bacterial metabolism.—In continuation of previous studies on the bacteriostatic effect of polyunsaturated fatty acids on Gram-positive bacteria, it was noted (196) that the inhibitory activity increased proportionally to the number of double bonds; the "all"-*cis* isomers were active, but *trans* isomers were not, thus simulating effects found in mammals by Privett *et al.* (386). Linoleate-adapted strains of *Lactobacillus casei* were also insensitive to oleic acid, partially to arachidonic, but not to linolenic acid. A physicochemical mechanism altering the permeability of the bacterial cell membrane has been proposed [Kodicek (196)]. A major constituent of the bacterial lipides appears to be the monounsaturated C_{18} component, *cis*-vaccenic acid, which is present in "bound" form [Hofman & Tausig (413, 414)].

Miscellaneous.—Tria (415) restated that the lipide-soluble unknown factor from liver which has a variety of metabolic effects is not identical with any of the known vitamins.

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NUTRITION[1]

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INTRODUCTION

It is axiomatic that success in research in any important field of investigation discloses unexplored areas inviting further study. Nutritional research is no exception. The remarkable progress of the past half-century in the understanding of the basic principles of human nutrition continues unabated but, as yet, there is no indication that a simple solution of many important and intriguing questions is at hand. Research in nutritional science is fostered by the growing realization of the great potentialities that exist in this field for the improvement of the health and well-being of mankind the world over. Although an appreciation of the need and a willingness to support investigations on soils, on agricultural production, on requirements of nutrients, and on the relation of food to health are not lacking, still greater attention is merited by the importance of the goals. The whole system of production, of processing, and of distribution of foodstuffs requires man's finest ingenuity and skill if the world's expanding population is to be nourished optimally.

The betterment of physical health through improved nutrition is possible in two ways, (*a*) by the administration of known nutrients in the form of analyzed foodstuffs, of enriched foods, or of supplements of the pure nutrients whose need has been demonstrated by appropriate tests and (*b*) by the increased use of specific foodstuffs whose value has been determined by experience rather than by experiment. The former method requires exact delineation of dietary requirements for optimal nutrition at all stages of human growth and development including senescence and provision of standards that allow the accurate assessment of nutritional status. Although progress is being made along these lines, it is disappointingly slow, and public health nutritionists are forced to depend on the second method all too frequently. Fortunately, there is an extensive background of experience at hand to show the way to good nutrition through a varied selection of foodstuffs. This is often a wasteful procedure, justified only by default.

To the difficulties arising from the lack of precise methods of detection of various grades of specific nutritional deficiencies may be added the disturbing claims of possible changes in the nutritive value of foodstuffs as a result of faulty fertilization of impoverished, deficient, or otherwise abnormal soils, to the use of deleterious chemical agents in the control of destructive

[1] The survey of the literature pertaining to this review was completed in November, 1955.

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agricultural pests, and to the manufacturing procedures utilized in the preparation of products that retain taste acceptability after prolonged storage. Gains in the quantity of available food are not necessarily gains in quality. Whether quantity or quality is the more important depends on the particular situation and the foodstuff in question. Difficult also are decisions on the suggested extension of enrichment programs to include additional foods and types of added nutrients and on the acceptability of proposals to substitute calorie-poor, nonnutritious substances, with or without added vitamins and minerals, for certain commonly used foods. In any case, reasonable decisions cannot be reached unless nutritionists keep abreast of the achievements of technological experts. It is increasingly evident that the public welfare demands the closest possible integration of advances in food technology and of determinations of safety and of nutritive quality of food products.

The complexity of the problem of making adequate food mixtures generally available is simplified only in part by the plentiful provision of foodstuffs of good quality and low cost. Each consumer must have the knowhow and the self-interest to select properly the kind and amount of the items in his daily fare. Increased knowledge about foods and about nutrition is imperative, therefore, if consumers everywhere are to profit from better eating practices. In this regard, nutritionists have a unique educational responsibility, one which requires judicious accuracy in the interpretation of dietary studies, and discerning realism in the application of experience.

These introductory comments on the status of problems of human nutrition in 1955 reflect the opinion of the reviewers that there is an increasing rather than decreasing need for (*a*) improved methods of measurement of nutritional status, (*b*) further developments in the production, processing, and distribution of foodstuffs, (*c*) more certain assurance of the safety and quality of foods, and (*d*) a broadened program of instruction in the knowhow of nutrition. Specific investigations are discussed under the following headings: requirements of amino acids and of proteins; role of carbohydrates and of fats; minerals; vitamins; chemical additives in foods and dietetic foods; and, nutrition surveys and special studies. The review has been restricted largely to the discussion of human nutrition.

AMINO ACIDS AND PROTEINS

Normal requirements of amino acids.—Values are now available for the minimum amounts of each of the essential amino acids required to maintain nitrogen equilibrium in healthy young adult men and women. Rose *et al.* (1 to 5) have published the details of some of their experiments relating to requirements in young men. Recently, Stiebeling (6) had reported studies by several investigators (7, 8, 9) on requirements in women. The results obtained in the two series of experiments are compared in Table I, with the highest observed values designated as minimum daily requirements. The general pattern of amino acid requirements in women is in many respects a remarkable confir-

mation of Rose's original observations. Nevertheless, the values appear significantly lower for women than for men, in the case of some amino acids at least. In these experiments there is no evidence that amino acid requirements are correlated with body size or body surface, so that divergent results are most likely to be explained on the basis of differences in sex or in diet composition. These investigations on amino acid requirements under strictly pre-

TABLE I

MINIMUM DAILY INTAKES OF ESSENTIAL AMINO ACIDS REQUIRED TO MAINTAIN NITROGEN BALANCE*

Amino Acid (L-form)	Minimum Daily Requirement	
	Men	Women
Isoleucine	0.70	0.45
Leucine	1.10	0.62
Lysine	0.80	0.40
Methionine	1.10	0.49†
Phenylalanine	1.10	1.12‡
Threonine	0.50	0.31
Tryptophan	0.25	0.16
Valine	0.80	0.65

* The values are expressed in grams.
† Part of this requirement was fed as cystine.
‡ Part of this requirement was fed as tyrosine.

scribed experimental conditions may have their greatest value in providing a starting point for a systematic study of factors that may cause variations in the protein requirements. Factors that should be studied include age, sex, and state of health, as well as the level of nitrogen and the amino acid pattern of the diet. Until these results are available, the translation of requirements of amino acids needed to maintain nitrogen balance into an over-all concept of nutritional requirements will be subject to modification.

Significant progress has also been made in defining the amino acid needs of infants. Pratt (10) and Snyderman (11) and co-workers have reported the phenylalanine and threonine requirements of infants fed the 18 L-amino acids in a mixture simulating the composition of breast milk. On the basis of nitrogen retention and weight gains the requirements have been set at 60 mg./kg./day for threonine and 90 mg./kg./day for phenylalanine. In an investigation to test if lysine was limiting in foods other than cereals, Albanese *et al.* (12) found that supplements of the amino acid in fresh or evaporated milk formulae increased the rate of gain in body weight and of nitrogen retention in 5 of 15 infants and enhanced nitrogen retention in an additional 6. These effects were observed in malnourished infants with poor appetites resulting possibly from a deficiency of digestive enzymes. Subse-

quent findings (13) indicated that optimum utilization of dietary nitrogen occurs if the lysine:tryptophan ratio of milk is made equivalent to that of mammalian muscle tissue. The suggestion that lysine deficiency may result from an abnormality in the digestive system in infants is important and needs further study.

Since attempts have been made to correlate blood and urinary levels of amino acids with nutritional status, two reports (14, 15) describing these levels under various experimental conditions are of interest. Doolan *et al.* (14) measured the renal clearance of 18 amino acids during continuous infusion of an amino acid mixture. Although less than 4 per cent of the total amount was excreted, there were greater variations than this in the excretion of individual amino acids. The results suggest that renal clearance is an important factor in determining the amount of a given amino acid excreted in the urine.

In another type of study of amino acid requirements, Eagle (16) has listed 13 amino acids as essential for the growth of a human epidermoid carcinoma in tissue culture. Further development of this technique may have far-reaching possibilities for the determination of amino acid requirements of specific body tissues.

In studies with individual amino acids, Rose & Wixom (17) found that 80 per cent of the methionine requirement in young adult men could be replaced by cystine, a much higher percentage than had been previously reported for the rat. Langner & Berg (18) showed that the ingestion of D-tryptophan, which is not utilizable by the human subject, is followed by its excretion in the urine as such, together with appreciable amounts of D-kynurenine. Reports (19, 20) also show that acetyl-D-tryptophan is not utilized by man and that acetylation of L-tryptophan does not interfere with its metabolic availability.

Requirements and effects of amino acids in disease.—Promising results have been obtained in the treatment of phenylketonurics (21, 22) by administration of a diet low in phenylalanine. Elevated blood levels of phenylalanine were lowered to normal on this diet and an increase in mental age was observed. The neurological changes were not as definite, but there was improvement, especially in younger children. The primary biochemical lesion causing impairment of the oxidation of phenylalanine is being studied in detail (23, 24, 25) and, as a result of the effects observed with a phenylalanine-free diet, it is postulated that the mental deficiency observed in phenylketonurics is attributable to intoxication by phenylalanine or one of its metabolites. It has also been noted (26) that the administration of tyrosine to phenylketonurics results in a darkening of the hair, indicating that tyrosine may not be available in optimal amounts to phenylketonurics in the usual dietary situation.

Kwashiorkor is another disease in which one causative factor, at least, may be undernutrition with respect to one or more amino acids. This widespread and serious condition is associated with diets deficient in animal pro-

tein and appears in an age group in which protein utilization is greatest, i.e., in infants (27). The subject has been capably reviewed by Brock (28) and included in his account is a very timely discussion of the whole problem of protein nutrition in the tropical and sub-tropical countries. The results of Brock and co-workers (29) showing the efficacy of dried skim milk in the treatment of kwashiorkor have now been published in detail. Recent studies have been concerned mainly with the more precise definition of the dietary deficiencies associated with malnutrition as it exists in the various countries and with effective methods of treatment. In the Belgian Congo a study (30) of nitrogen and fat absorption in infants with kwashiorkor revealed a high fecal nitrogen excretion but fat absorption was within normal limits. In India (31) a follow-up study of 100 cases of kwashiorkor revealed no improvement in dietary or environmental conditions although instructions had been given to the patients. Thirty-two per cent of the cases relapsed, 36 per cent died, and 50 per cent of the survivors showed signs of nutritional deficiency. Supplementation of the vegetable diets of South America with lysine and methionine was thought by Hegsted and others (32) to be of some value. In another study in South America (33) the substitution of a vegetable diet for a mixed diet containing animal protein had no immediate effect on the growth and weight of the 23 children used in the experiment. Trowell (34) states that dietary surveys in Africa reveal a low intake of both calories and proteins and poses the question whether Africans eat less because they work less or vice versa. An investigation (35) of the daily intake of food in a boarding school in the Uganda gave no evidence that the children were undernourished, although all food intakes were below 90 per cent of those of British boys, and the children were underweight by British standards. It was strongly urged that, before any type of supplementation is instigated, controlled experiments should be carried out to determine whether it is advantageous. Dean (36) points out that protein consumption is influenced by many factors: dislike of new foods, cost in relation to protein content, and utilization by recipients. Autret & van Veen (37) have surveyed the food sources that could be used for supplementation in underdeveloped countries.

A complete description of kwashiorkor, as it occurs in Central America where it is known as the "infantile pluricarencial syndrome," is given by Scrimshaw and others (38) in a recent review of nutritional problems in that area. Many clinical cases of the disease were noted. Dietary surveys showed basic deficiencies of good quality protein, of vitamin A, and of riboflavin. In addition, moderate protein deficiency was considered of common occurrence. School children from low income families were two years behind the Cleveland standards in skeletal development, with parallel retardation in height and weight. Continuation of extensive studies will not only benefit enormously the population groups in Central America but should also yield valuable data that will aid in distinguishing racial and genetic differences from purely nutritional factors.

Walters & Waterlow (39) have studied fibrosis of the liver in West African

children in the Gambia. Malnutrition was common, the average caloric intake varying from 1570 to 1980 at different seasons of the year with a 50 gm. intake of protein of vegetable origin. Fatty infiltration of the liver was uncommon at any age, hence the diet was not lacking in lipotropic factors. However, there was a high incidence of parasitic infestations, especially of the malarial organism, and the investigators proposed that malaria evoked a localized inflammatory reaction in liver tissue which proved harmful in the protein-deficient child. It was concluded that malaria and malnutrition both contributed to the fibrotic liver.

The optimum intake of protein in subjects with liver disease remains to be determined. In controlled observations on the treatment of acute infectious hepatitis, Chalmers and others (40) found that patients forced to eat a high-calorie and high-protein diet improved more rapidly than patients eating what they chose of a normal diet. Two reports (41, 42) indicate that excess dietary protein may precipitate coma in patients with liver disease. Amino acid levels are high in hepatic coma, and it has been found (43) that glutamic acid, glutamine, and tyrosine were increased in 9 out of 12 patients, with cystine and methionine also showing increases in the majority. Ammonia as the cause of hepatic coma has been studied by Bessman & Bessman (44) who postulate that it interferes with the Krebs cycle in the brain by reversing the glutamic dehydrogenase reaction with formation of glutamate and reduction of available ketoglutarate. White *et al.* (45) indicate that in liver disease either the liver is unable to metabolize ammonia normally or portal collateral veins divert it from the liver so that it escapes metabolism. The distinction between choline-deficient and protein-deficient fatty livers in rats is emphasized by Best *et al.* (46). Fat of the livers of protein-deficient animals is periportal and the accumulation is not so massive as that which occurs in the cells bordering the central vein in choline deficiency.

Protein nutrition in severely burned patients was studied by Blocker and associates (47, 48) using S^{35}-labeled L-methionine. Incorporation of the amino acid in serum proteins was accelerated during the early period following the burn injury, suggesting a stimulation of protein anabolism. Catabolism also proceeded at a greater rate as shown by increased urinary nitrogen excretion. The increase in urinary nitrogen was less in patients whose dietary intakes were maintained at relatively high levels.

Nisensen (49) found hypoproteinemia in 20 of 24 children with eczema and noted a correlation with the use of vegetable protein alone in the diet. Illingworth and co-workers (50) were unable to demonstrate any advantage in protein restriction in acute nephritis.

The use of gluten-free diets in the treatment of celiac disease represents an important advance in the definition of the etiology of this condition and implicates a protein as the causative agent. The original observations in 1950 of Dicke (51) and of Weijers & van de Kamer (52) have now been extended and confirmed (53 to 56). Ross and others (55), found that 28 of 30 cases with active celiac disease responded favorably to diets devoid of

wheat gluten, as evidenced by a decrease in fecal fat excretion. To prove that wheat gluten was the causative factor, the protein was reintroduced into the diet of 12 of these children, and all developed steatorrhea in two to four weeks. Thirty-two children and adolescents who were diagnosed as having celiac disease in early childhood but who had not been treated with the gluten-free diet were placed on the regimen for prolonged periods of time. After 4 to 25 months on the diet most of the subjects showed an acceleration of growth in weight and height. Van de Kamer & Weijers (57) have recently proposed that the harmful effect of the specific protein, wheat gliadin, in celiac disease is attributable to its high percentage of bound glutamic acid. These workers were unable to produce steatorrhea in two patients with celiac disease by administration of deamidated gliadin. A working hypothesis was presented in which the deleterious effect of various proteins in celiac disease is correlated with the amide nitrogen content of the protein.

The investigations cited above indicate current interest in the role of individual amino acids as essential components of the diet. The results of the past two decades, in particular, represent great expenditures of research effort and are unquestionably highly important contributions to the biochemistry of nutrition. Inevitably, however, the studies on amino acids serve to emphasize how fragmentary is our knowledge of the details of the nutritional aspects of protein or nitrogen metabolism. There is growing realization of the necessity of a much clearer understanding of the metabolic interrelationships between the individual amino acids and between amino acids and other nutrients. Not only is it essential to know the requirement of amino acids at all ages but, in addition, the influence of varying conditions of health and of environment. It is important to learn, for instance, if the findings of Rose represent desirable amino acid intakes for young men on diets differing from those used in the experimental studies. What are the influences of variations in the amount of dispensable nitrogen, of folic acid and of vitamin B_{12}, of illness and of previous protein malnutrition? Does the absorption of even trace quantities of peptides formed in the normal digestion of protein in the alimentary tract modify the efficiency of use of amino acids? How important is the rate of liberation of amino acids from dietary protein in digestion and the rate of absorption into the bloodstream, especially in disease or in other abnormal conditions? Is it possible that protein synthesis in tissues is not necessarily more efficient merely because of the mass action effect of simultaneous excesses of amino acids accumulating at sites of protein formation following their oral or parenteral administration? Significantly, evidence of interference of one natural amino acid with another, the two being leucine and isoleucine, has been reported (58, 59). These and many other problems remain to be solved.

Biological value of proteins.—Inasmuch as there appears neither desirability nor possibility of a replacement of dietary protein by amino acids in any normal diet, the measurement of biological value of food proteins under various circumstances continues to be important. An excellent review of this

subject by Allison (60) has appeared. Allison lists the following procedures for estimating the relative efficiency of individual proteins or of protein-containing foods in supplying the body's need of amino acids: (*a*) growth of young animals, usually rats; (*b*) repletion of depleted adult animals, usually rats; (*c*) nitrogen balance in rats, dogs, and man; (*d*) filling the blood protein compartment, usually in dogs; (*e*) filling the liver protein compartment, usually in rats, with or without the determination of levels of specific enzymes of liver and other tissues; (*f*) growth of protozoa and other micro-organisms; and (*g*) quantitative chemical or microbiological determination of the amino acids in proteins. Allison properly emphasizes the need for more studies of the biological value of proteins in animals in conditions of stress. This is especially true for man in whom controlled experimentation during illness, trauma, and hormonal imbalance is urgently required.

The determinations listed above do not measure necessarily any single nutritive value of a protein. In the case of man, the most informative procedures are the measurements of true growth in infants and older children, and of the efficiency of utilization of absorbed nitrogen in adults. The percentage retention of absorbed nitrogen as a measure of biological value of protein was first formulated by Thomas (61) and extensive subsequent studies, especially those of Mitchell and of Allison, have served to emphasize the following formulation of his concept of biological value:

$$\text{Biological Value} = 100 \times \frac{\begin{matrix}\text{Nitrogen} \\ \text{Balance}\end{matrix} + \begin{matrix}\text{Metabolic} \\ \text{Fecal N}\end{matrix} + \begin{matrix}\text{Endogenous} \\ \text{Urinary N}\end{matrix}}{\begin{matrix}\text{Nitrogen} \\ \text{Intake}\end{matrix} + \begin{matrix}\text{Metabolic} \\ \text{Fecal N}\end{matrix} - \begin{matrix}\text{Total} \\ \text{Fecal N}\end{matrix}}$$

The numerator and denominator are definitions of retained and absorbed nitrogen, respectively. The constancy of the metabolic fecal nitrogen, the nitrogen entering the alimentary tract from digestive and other tissues, is generally assumed. Whether the urinary nitrogen on a protein-free but otherwise adequate diet is a true measure of endogenous nitrogen metabolism is controversial. The present reviewers agree with Mitchell (62) that no well-defined findings in the area of protein metabolism have been found contradictory to the concept of a type of protein metabolism that is independent of protein intake except as the latter determines the protein stores of the body. At any rate, the measurement of biological value of proteins according to nitrogen balance data obtained under strictly controlled and reproducible conditions provides valuable and useful information.

The difficulty in the determination of the relative value of proteins and of protein mixtures in man is responsible for accelerated efforts to find simpler procedures that may replace nitrogen balance studies. This is an especially urgent problem because of the necessity of having definite knowledge of the extent of losses of nutrient value in processed foodstuffs as a result of rigorous stabilizing procedures or long storage. Determination of the amino acid make-up of proteins is an appealing method of assessment of biological value now that sound analytical methods and reliable data on the

requirement of indispensable amino acids are available. Table II, taken from Mitchell (63), shows a reasonably good correlation between the biological values and the essential amino acid indices of typical proteins. Unfortunately the laboratory analysis of protein does not reflect the possible influence of factors that change digestibility and absorption. Present evidence supports the conclusion that the proportionate requirements of essential amino acids for the maintenance of nitrogen equilibrium in man under normal conditions simulate the requirement of growing animals for the maintenance of maximum nitrogen retention.

Supplementation of proteins with amino acids is a certain method of improving the biological value if the resulting mixture more closely satisfies the total requirements. Sure (71) has shown, for instance, that growth of young rats is greatly increased by supplementing milled barley protein

TABLE II

Correlation Between the Biological Values of Food Proteins for Adult Man and their Modified Essential Amino Acid Indices (EAAI)*

Protein Source	Modified EAAI	Biological value	Food source	Modified EAAI	Biological value
Whole egg	100	91	Yeast	81	52
Milk	94	74	Beef muscle R	78	67
Egg albumin R	93	91	Sunflower seed	73	62
Whole egg R†	87	94	Wheat gluten R	62	42
Casein R	87	68	White flour	60	41
Soy flour	84	65	Peanut flour R	56	56

* From data of Mitchell (63).

† The whole egg preparation in the Rutgers study was definitely impaired in biological value for the growing, as well as the mature, rat (64).

Note. Those foods designated R were those tested in the Rutgers cooperative study (65). The EAAI were computed from the analyses given in that report and the corresponding biological values are those reported by Hawley *et al.* (66). All other indices were computed from analyses given by Block & Bolling (67). Biological values other than those of Hawley *et al.* are taken from Bricker, Mitchell & Kinsman (68): milk, soy flour, and white flour; from Bricker & Smith (69): milk and sunflower seed flour; and Cremer & Lang (70): yeast.

with lysine, threonine, and methionine, and peanut protein with methionine and threonine. The desirability of this procedure in foodstuffs used in animal rations and in human dietaries will be discussed later.

Endogenous protein metabolism.—Reference has been made to the lack of unanimity on the significance of endogenous protein or nitrogen metabolism. Continuing interest in this fascinating problem is not surprising because endogenous, or wear and tear and erosion processes have never been satisfactorily clarified. In the absence of explanatory data it is difficult, in-

deed, to think of the wearing out and necessary replacement of a red cell and of its protein, for instance, in the same way in which one considers the replacement of the lost keratin of hair, nails, and skin. Does the body conserve the amino acids of disintegrated globin and stroma protein as it does iron or is the so-called wear and tear metabolism really a convenient way to refer to small but unavoidable losses of amino acids in addition to the more significant excretion of nitrogen as creatinine? What is the relationship of the level of protein stores to endogenous metabolism?

The failure to locate and to identify a special storage protein, analogous to liver glycogen or to adipose tissues, has also confused the general understanding of endogenous and exogenous metabolism. That there is a form of storage of protein in the body seems certain. Whipple referred to this as deposit protein; Allison has written of the filling of the blood or tissue compartment with protein; and others, including Mitchell, have used the unequivocal term, protein stores. A second source of confusion is the difficulty in reconciling what appear to be contradictory conclusions concerning the influence of protein stores on the excretion of endogenous urinary nitrogen. One finds, for instance, in the paper by Mitchell (62), the apparent acceptance of Allison's belief that the level of excretion of endogenous urinary nitrogen is correlated with the protein stores in the body, being reduced with a decrease in protein stores as measured by the blood plasma level. Yet, Mitchell concludes that his own data show that a "constant fraction of the absorbed nitrogen above the nitrogen output on a nitrogen-free diet is retained in the body of the growing animal, implying strongly that the endogenous output of urinary nitrogen continues at a constant level throughout subsequent periods of increasing protein intake." It would appear that such periods of increasing protein intake should increase protein stores and, therefore, increase the level of the urinary excretion of endogenous nitrogen. Possibly the term, protein stores, is defined differently by Mitchell and by Allison.

Mitchell has recently presented additional evidence of the real distinction between endogenous and exogenous nitrogen metabolism, as originally presented by Folin. Many investigators have assumed that the brilliant isotopic studies of Schoenheimer and of later workers made necessary the discarding of Folin's concept of two different types of nitrogen metabolism, the one constant and the other variable. This is far from true because the isotopic studies support, in fact, a difference between the relatively static nitrogen metabolism of fixed protein tissues and the dynamic activity of the "ebb and flow" deposit type of protein which is a direct reflection of the protein intake.

CALORIC BALANCE, FATS AND CARBOHYDRATES

The proper assessment of the energy values of human and animal foods was the subject of several papers presented to the British Nutrition Society (72, 73). The most recent tables of food composition published by the Food

and Agriculture Organization gave caloric results 3 to 4 per cent higher than the Medical Research Council Accessory Food Factors Committee of Great Britain. This difference becomes significant when the caloric consumption of nations or large population groups is being analyzed. A handbook on the basis and derivation of the energy value of foods has been issued by the United States Department of Agriculture (74).

In a study of individual energy expenditure, by Passmore and others (75, 76), three thin young men were overfed from 1300 to 1600 calories for periods of 10 to 14 days. The gain in weight was less than three fourths of the gain expected, and there was no evidence of gain in body water. The investigators concluded that the subjects stayed thin despite overfeeding because they concentrated their tissues in both fat and protein. The factors responsible for individual variations in cellular fat and protein appear to be entirely unknown. Edholm and co-workers (77) measured the energy expenditures of 12 young cadets in training and found wide variations from day to day and also from one individual to another. There was no correlation between the expenditure and intake on the same day, but there was a significant correlation with the intake two days later. The belated adjustment of intake of food to correct for expenditure of energy could not, in the opinion of the investigators, be adequately explained by theories relating appetite to the level of some circulating metabolite such as glucose. Other experiments on relations between body weight, body fat content, caloric intake and expenditure of energy have been carried out by Grossman & Sloane (78), Johnston & Bernstein (79) and Miller & Blyth (80).

The effect of climate on caloric intake was studied by Stadler & Grossman (81) who concluded that troops in garrison in the sub-arctic performing normal duties, require no more than 3500 calories in summer or winter, if accustomed to the climate and if excessive dissipation of body heat in the cold environment is prevented by protective clothing. This is not significantly different from amounts previously found for temperate climates. Buskirk and others (82) measured the work performance of 13 men kept for 24 days on a 1010 calorie carbohydrate diet and found that, although there was an average weight loss of 7.5 kg., the diet caused only a small impairment of the capacity to perform physical work.

Widdowson (83) has reviewed obesity in relation to reproduction. Master & Jaffe (84) studied the incidence of obesity in 1000 cases of coronary disease and found a higher percentage of overweight men than in the general population. However, this was not true for women. The significance of the method for determining obesity by skin fold measurement as it applies to the different racial groups was studied by Garn (85) and Newman (86). Negroes appear to have a different fat distribution pattern.

In regard to the problem of obesity, the theory of the glucostatic control of hunger has been reviewed by Mayer (87). Two laboratories have reported experiments testing the glucostatic theory of Mayer and associates (88), which relates appetite and hunger to the difference in glucose level between

arterial and venous blood. No confirmation was obtained in either case. Fryer and associates (89, 90) worked with 12 overweight subjects fed isocaloric diets in which the proportion of carbohydrate, fat, and protein varied and found no correlation between satiety value of the diets and the arterial-venous difference of blood glucose. Since the subjects were overweight, these results may not apply to individuals of average weight. It is of interest that the low protein diet was rated as having the lowest satiety value. Grossman & Bernstein (91, 92) gave normal male subjects either glucose or saline solutions intragastrically or intravenously just before a test meal was eaten ad libitum. Hyperglycemia did not depress hunger sensations, appetite, or food consumption. In another type of experiment, Stunkard, Van Itallie & Reis (93) studied the effect of intravenous glucagon on the gastric contractions of seven normal subjects in the fasting state. Three minutes after the glucagon injection, gastric contractions stopped and the sensation of hunger also disappeared. Arterial-venous glucose levels were not reported in this experiment, but further studies relating to glucagon and hunger will be awaited with interest.

Current concepts on the metabolism of fructose have been summarized by Renold & Thorn (94). Fructose differs from glucose in that it is utilized almost exclusively by the liver, separate enzymes are used, and there is no dependence on insulin. Its possible clinical uses and dangers are noted.

The effect of diet on the level of blood lipides continues to be the subject of numerous reports. Keys and co-workers (95) reviewed the evidence obtained in several population groups for a correlation between the proportion of fat in the diet and the concentration of blood lipides in the serum. Serum cholesterol levels of 46 Nigerian men (96) were significantly lower than those in an age and weight matched group in the United States. Serum lipoproteins were only slightly lower. It was found by dietary survey that Nigerians consume diets low in fat and carbohydrate.

Mann (97) studied the effect of the combination of exercise and high calorie intakes on serum lipide levels in three healthy men. The subjects were fed a control diet and then a diet containing twice the calories, but with the same fat content as the control diet. When the caloric intake was raised, the serum level remained constant as long as exercise dissipated the excess calories. When exercise was reduced and weight increased, the serum cholesterol content rose to almost twice the original value, and serum lipoproteins and phospholipides also rose in two subjects. On the basis of these observations and others, including the Nigerian survey, it was suggested (96) that low serum cholesterol levels may be directly related to muscular activity and energy expenditure. It would appear that this hypothesis should be studied further.

Experiments have also been reported showing the effect of the type of fat fed on the serum lipide levels. Beveridge and others (98) found that the administration of vegetable fat in a homogeneous formula diet lowered plasma lipide levels whereas substitution of animal fat increased the lipide

level from a control period value. Ahrens and co-workers (99) also found a reduction in lipide level following the feeding of vegetable fats. A corn oil formula gave the lowest values. Kinsell & Michaels (100) have reported results for one patient showing that the feeding of isocaloric amounts of various vegetable fats resulted in decreases of plasma cholesterol that were proportional to the unsaturated fatty acid content of the fat.

Nicotinic acid (101) was reported to lower serum cholesterol, whereas the lipotropic agents, choline and inositol (102, 103), and sitosterol and similar compounds (104, 105) were ineffective. In the first study (104) unpurified soybean sitosterol (presumably gamma sitosterol) was used. Best *et al.* (106) found a consistent and sustained lowering of serum cholesterol levels after prolonged administration of beta sitosterol. In 9 of 10 fasting subjects there was a rise in the free fatty acid content of whole serum after the injection of heparin (107). The complete elimination of fat from the diet reduced the cholesterol level by 15 per cent according to Hatch and others (108).

Blood lipide levels have been studied in relation to weight reduction by Moore and colleagues (109). It was concluded that the relationship between the level of blood lipides and weight loss was not of physiologic significance. Serum cholesterol levels in relation to age were studied in 184 women by Swanson and others (110). The levels increased with age from 163 in the 30 year decade to 260 mg. per cent in the 60 year decade and then declined. In a similar study (111) with 58 women, serum cholesterol levels also increased with age to the 70 year decade and then declined. Unsaturated fatty acids in the blood plasma of atherosclerotics were measured by Hammond & Lundberg (112). In eight subjects the levels of di- and tetraenoic acids were lower than for blood bank donors, but the trienoic acid levels were higher. Unsaturated fatty acid blood levels were also studied in infants by Hansen *et al.* (113) who found that breast-fed infants tended to have higher blood levels of di- and tetraenoic acids than infants fed evaporated milk formulas. On a low-fat diet serum levels of di- and tetraenoic acids decreased.

Regarding absorption of fats, it was found (114) that premature infants who have difficulty absorbing dietary fat absorb tributyrin and triacetin. However, the low energy value of these substances makes the observation of little practical use. French (115) has reviewed the disorders of the alimentary tract related to fat absorption. The beneficial effect of a gluten-free diet on the steatorrhea of celiac disease indicates a positive causal factor in a condition previously thought to be a deficiency disease. In view of this finding a reconsideration of the theories concerning the production of adult steatorrhea is suggested.

The intravenous infusion of fat emulsions continues to be studied with promising results (116, 117). In a series of 426 patients 80 to 90 per cent of the infusions were without adverse reactions.

A symposium on lipides in nutrition included reviews on the nutritive

value of fats (118), on the evaluation of fat soluble vitamins (119), on the effect of various lipides in experimental hypolipotropic diets (120), and on hormone-lipide relationships (100).

MINERALS

A trend in present-day nutrition is to give greater consideration to the importance of the mineral elements whatever the organism under study (121).

Iodine.—A most interesting report has been made with respect to iodine. Hamwi and co-workers (122) examined over 27,000 children for enlarged thyroid. The survey took place in Ohio and included the same counties that participated in the 1925 experiment which established the therapeutic effect of iodides in goiter. The incidence of enlarged thyroid has dropped from 32 per cent in 1925 to 4 per cent in 1954. The decline was not attributed solely to use of iodized salt because the occurrence in children from homes not using the enriched salt was only 6.06 per cent. The decline was partially attributed to the increased use of seafoods and to milk and eggs from animals fed rations grown in states where the soil contained iodine. It was concluded that endemic goiter is still a minor health problem in Ohio.

Bishopric, Garret, & Nicholson (123) have studied the thyroidal uptake of I^{131} in a series of 20 patients who had been on a rice diet regimen. They found a high iodine uptake in many euthyroid patients after they had been on the diet for a three month period and suggested the effect was attributable to a dietary restriction of iodine. These results present the possibility that thyroidal uptake of I^{131} might be used to detect subclinical iodine deficiency states.

Calcium.—Of the recommended allowances for the various nutrients proposed by the National Research Council in 1953, the calcium allowance was the subject of most discussion (124, 125). The use of balance studies as a means of determining the requirement of calcium has been criticized, and there is no definitive test for calcium nutrition in the adult.

The utilization of calcium salts by college women (126) and by children (127) has been studied. Meyer, Kyle & Schaaf (128) measured calcium retention following an intravenous infusion of calcium and found the same amount of retention in normal and osteoporotic subjects. During the healing phase of osteoporosis in Cushing's syndrome there was an increase in calcium retention.

Several reports (129, 130, 131) have appeared on what is termed the milk-alkali syndrome which occurs occasionally in peptic ulcer patients. The symptoms, hypercalcemia, renal insufficiency, and azotemia, are similar to those produced under other circumstances, such as hypervitaminosis D. The symptoms disappear when milk and antacids containing absorbable alkali are omitted from the diet. Harris (132) has reemphasized the interference of phytic acid with calcium availability in areas in which there is a high consumption of unrefined flour and a low consumption of calcium-containing foods.

The role of dietary factors other than calcium in the formation and preservation of tooth structure continues to be a perplexing problem. In a study (133) of 442 Guatemalan children from 6 to 14 years of age, a very low incidence of dental caries was found despite a retardation of one year in bone age as compared with children in the United States. The diet was described as deficient in vitamin A, riboflavin, and animal protein and extremely low in refined carbohydrate. A study of children in Tel Aviv (134) also showed a low incidence of dental caries, and the diet was suggested as the responsible factor. No significant effect on the development of dental caries during a two-year observation period was noted in a group of London children given a supplement of sugar (135). The pros and cons of fluoridation of drinking water are reviewed by Jenkins (136). A progress report on the Evanston dental caries study (137, 138) gives the caries experience rates of 12-, 13-, and 14-year-old children after exposure to fluoridated water for 59 to 70 months. The reduction rate in dental caries was found statistically significant and to average 4 per cent for each year of fluoridation. The topical application of fluoride gave no reduction in caries incidence among 60 adult females (139), but the use of a dentifrice containing stannous fluoride for six months was reported to reduce the dental caries in a group of 6- to 15-year-old children, with greater protection in the older age group (140). Fluorine added to the drinking water had no effect on the thyroidal uptake of I^{131} (141).

Iron.—In studies relating to iron nutriture, Smith *et al.* (142) measured the iron content of 16 infants whose mothers had received red blood cells containing Fe^{55}. The results showed that the normal infant utilized iron obtained during fetal life throughout infancy and incorporated significant amounts of dietary iron only after three to four months of age.

In well-planned experiments using natural foods containing Fe^{59} it was found by Moore and collaborators (143, 144) that healthy adults absorb on the average about 10 per cent of the iron in their diet, and iron-deficient patients usually absorb more. Ascorbic acid increases the assimilation of food iron. The iron lost by the body in the excretions was also determined using Fe^{59} given by injection as ferrous ascorbate in four normal individuals (144). Fecal specimens were collected for 140 days and the radioactivity measured. The radioiron was also determined in sweat during periods of induced perspiration. The investigators concluded that the total amount of iron excreted by the body each day in the absence of bleeding averages between 0.5 and 1.0 mg. On the basis of these studies Moore (143) states that if a purely nutritional iron deficiency ever occurs in adult men or postmenopausal women, many years would be required for its production.

In a discussion of the high serum iron values and of the abnormal deposition of iron in the tissues of the adult Bantu, Walker (145) noted that hypochromic anemia is extremely rare. These individuals are apparently habituated to a very high level of iron intake, as a result of the use of iron cooking utensils. The effectiveness of various iron salts used in the fortification of flour was studied by Steinkamp *et al.* (146). No significant differences were found in human volunteers in the utilization of the iron in bread sup-

plied by supplements of Fe^{59} contained in ferrous sulfate, reduced iron, ferric orthophosphate, and sodium ferric pyrophosphate.

Copper and cobalt.—Cartwright has reviewed the nutritional aspects of copper and cobalt (147). The daily adult requirement for copper was estimated as 2 mg. per day, with the diet supplying from 2.5 to 5 mg. per day. He measured the plasma copper levels in 228 normal individuals and 300 patients with various disorders and found no evidence of copper depletion.

The only known physiologic role of cobalt in hemopoiesis is as a component of the vitamin B_{12} molecule. Since man requires a dietary source of vitamin B_{12}, a cobalt deficiency would not be expected to have any effect on hemopoiesis in human nutrition. Davis & Fields (148) have reported the experimental production of polycythemia in four young men ingesting daily doses of 150 mg. of cobaltous chloride. This stimulating effect of cobalt on erythropoiesis also occurs in animals and has been studied by several investigators, but the mechanism is unknown. The use of cobalt in the treatment of various refractory anemias has been reviewed by a panel of hematologists (149). In the opinion of many, the widespread use of this potentially toxic compound is not justifiable before more adequately controlled studies have been carried out.

Sodium and potassium.—As a result of the increasing use of the low-sodium diet in medical therapeutics, the National Research Council has published a brochure describing the rationale, complications, and practical aspects of this type of diet (150). Hulet (151) has determined by actual assay the sodium content of 13 different diets presumed to contain 200 mg. of sodium. The average figure obtained was 513 mg.

There have been reports on the effects of low potassium and sodium diets (152, 153), and a deficiency syndrome resulting from potassium depletion (154, 155) has been described. In five adults with a severe chronic potassium depletion a renal disorder was discovered in one, and renal insufficiency and an acute degeneration of skeletal muscle occurred in another. Dietary treatment with potassium salts resulted in restoration of normal renal and muscular function.

VITAMINS

Vitamin B_{12} and folic acid.—Current research on vitamin B_{12} continues to have important implications for human nutrition. Two phases of this research, the chemotherapeutic action of vitamin B_{12} and its role in the metabolism of microorganisms, are very capably reviewed in *Vitamins and Hormones* for 1955 (156, 157). It is now unquestioned that vitamin B_{12} is an essential nutrient for man, but thus far the minimal and optimal requirements have not been established. However, our knowledge has advanced to the point where the problems concerning standards can be considered. Many of these problems relate to the unique characteristics of vitamin B_{12} that distinguish it from other members of the vitamin B complex: it is not

found in plant tissue; it is absorbed from the intestine in limited amounts and under special conditions; and it is stored in the body to a considerable extent.

Although vitamin B_{12} is present in the large intestine as a result of bacterial synthesis, Hausmann and others (158) have provided evidence that it is not directly absorbed by the body. This indicates that the chief source of the vitamin for man is dietary in nature and, since vitamin B_{12} is not present in plant material, it would appear that vegetarians who exclude animal food from their diet should develop a deficiency. Wokes, Badenoch & Sinclair (159) have reported on the dietary status of a group of "vegans," individuals who have lived entirely on vegetables without milk or eggs included in their food. Symptoms of ill health did not appear for several years, and children were more susceptible than adults. The commonest symptoms were a transient sore tongue, paresthesia, and other complaints referable to the nervous system. No frank anemias were found, and it was suggested that the high folic acid content of the diet prevented their development. Low serum vitamin B_{12} levels (a range of 0.045 to 0.193 $\mu\mu$g. per ml.) were the most definite evidence of a deficiency of this vitamin. The investigators point out that the nutritional status of the vegans has not been determined in regard to other vitamins, minerals, and amino acids. Although some of the children grow and develop satisfactorily for several years on the diet, it is pertinent that vegan mothers keep them on breast milk for long periods of time. Wokes & Picard (160) reported an increased urinary excretion of thiocyanate in vegans which appeared to be correlated with lowered vitamin B_{12} serum levels. Pollycove and others (161) studied a man of 60 who had avoided all foods of animal origin for eight years. This individual had a serum vitamin B_{12} level of 0.07 $\mu\mu$g. per ml. and a macrocytic anemia which responded to parenterally administered vitamin B_{12}. By studies with Co^{60} vitamin B_{12} it was shown that absorption from the gastrointestinal tract was within normal limits. This case appears to be a very clear cut example of a vitamin B_{12}-deficiency induced by a dietary lack.

There is ample evidence that absorption defects can also produce a vitamin B_{12} deficiency (156). The absorption of vitamin B_{12} when ingested in physiologic amounts is dependent upon the presence of an intrinsic factor elaborated by the gastric mucosa. In pernicious anemia and in total gastrectomy the absorption of vitamin B_{12} does not occur unless an intrinsic factor source is given or relatively large amounts of the vitamin are administered so that a small portion diffuses across the intestinal barrier. Absorption of vitamin B_{12} is also impaired in such conditions as sprue, the blind intestinal loop syndrome, and infestations with fish tapeworm. A report by Callender & Evans (162) confirms previous studies that the intestine has a limited capacity to absorb vitamin B_{12} even in the presence of intrinsic factor, the upper level of absorption being from 0.7 to 1.3 μg. It is not known whether this level is exceeded in a vitamin B_{12} deficiency state. It is not increased by exogenous supplements of intrinsic factor. It is also not known with cer-

tainty whether intrinsic factor acts by combining with vitamin B_{12} or exerts its effect on the intestinal wall. Baker & Mollin (163) show that a stoichiometric relationship exists between the vitamin and intrinsic factor over a limited range. Rosenblum and co-workers (164) using Co^{60}-labeled material have shown that chlorocobalamin is less well absorbed than cyanocobalamin. There is also evidence that pseudovitamin B_{12} is not absorbed (165). Apparently, alterations in molecular structure affect the passage of the vitamin across the intestinal barrier. The first report has appeared (166) of a homogeneous material isolated from a human source that serves as intrinsic factor. This substance has different physico-chemical properties from material previously isolated from hog mucosa. As Latner has pointed out (167) intrinsic factor may have some degree of species specificity and may vary in its ability to promote absorption of vitamin B_{12}. As yet no substance other than from a gastric or duodenal source has been shown to possess intrinsic factor activity. One wonders whether some of the vitamin B_{12}-binding substances might have this ability under special circumstances. For example, it is possible that the vitamin B_{12}-binding material in mother's milk may have the function of promoting vitamin B_{12} absorption in the infant.

It does not appear feasible to attempt the experimental production of a vitamin B_{12} deficiency in humans because of the length of time necessary to deplete body stores, as shown by the reported cases of the "vegans." Patients with pernicious anemia develop a vitamin B_{12} deficiency as a result of an absorption defect. It would appear, therefore, that the maintenance requirements of vitamin B_{12} given by injection to these patients would be a fairly accurate reflection of the requirements of normal individuals. Several studies on the treatment of pernicious anemia with vitamin B_{12} have been reported (168, 169, 170). The requirements of pernicious anemia patients for injected vitamin B_{12} have been estimated to be between 1 and 2 μg. per day (171). Using this amount and from values obtained by measurement of vitamin B_{12} in liver tissue (172), it can be calculated that the body stores are sufficient to last for several years.

From the limited evidence available it appears that the symptoms associated with a vitamin B_{12}-deficiency may vary depending on the presence of other nutrients in the diet. In particular, folic acid supplements may prevent the development of macrocytic anemia (173). The degree of anemia is apparently not a reliable index as a criterion to assess the nutritional status of vitamin B_{12}, but the serum vitamin B_{12} level might be a good criterion (174). Several investigators have found low values in untreated pernicious anemia patients (157). Girdwood (175) and Pitney & Beard (176) have also found low serum vitamin B_{12} values in patients who have had gastrectomies. For one such patient, concentrations in the serum were determined at various time intervals after the operation. After 20 months the serum vitamin B_{12} level had fallen from 0.150 to less than 0.050 $\mu\mu$g. per ml. Although the absorption of vitamin B_{12} is limited by the intestinal

barrier, after injection the vitamin is readily excreted by the kidney. Some modification of a vitamin B_{12} tolerance test based on urinary excretion following intramuscular injection such as that used by Estrada and others (177) might be of value in assessing vitamin B_{12} nutriture. Although vitamin B_{12} deficiency in the United States and many other countries may be limited to individuals with absorption defects, in other areas of the world where animal food is not in plentiful supply there is the possibility of widespread dietary deficiency of vitamin B_{12}. The development of suitable criteria for measuring the nutritional status of this vitamin is therefore of real significance.

Studies on vitamin B_{12} nutrition in old age show a slight decrease in serum levels (178), no change in liver stores (172), and an increase in absorption following the administration of intrinsic factor supplements in individuals with gastric anacidity (179). Serum vitamin B_{12} levels averaged higher than normal in patients with liver cirrhosis (180), but in another series of patients it was found that liver stores were greatly reduced (181). Several investigations were carried out on the effect of vitamin B_{12} supplementation on the growth of children. No effect was reported in most of the studies (182 to 185). Jolliffe (186) in a study carried out on a group of school children in Italy showed that supplementation with 20 μg. vitamin B_{12} per day caused a statistically significant increase in weight.

The effect of folic acid and vitamin B_{12} administered singly or in combination in the various megaloblastic anemias continues to be studied (187, 188, 189). However the nutritional relationship of these vitamins will not be clarified until their exact metabolic role is better defined. The indiscriminate use of folic acid supplements is potentially hazardous for individuals who may have pernicious anemia because it permits neurologic degeneration to occur while preventing the development of anemia which is often the diagnostic sign (190).

Niacin.—Goldsmith and others have proposed a procedure for assessing the nutritional status of niacin (191) based on measurement of urinary excretion of niacin metabolites. A standard diet containing 10 mg. of niacin and 1 gm. of tryptophan is given for 24 hr., and the excretion in the urine of N^1-methylnicotinamide and of its pyridone is measured. Excretion in cases of niacin deficiency is much lower than in normal subjects. The same investigators have continued their studies (192) of the niacin requirement on wheat and corn diets low in tryptophan. In a previous study three subjects on the corn diet developed the clinical picture of niacin deficiency after 50 days. Of three subjects on the wheat diet, one developed deficiency symptoms after 80 days. Since the tryptophan content of both diets was similar, it was suggested that the low tryptophan content of corn might not be the sole explanation of its pellagragenic effect. When the corn diet was supplemented with varying amounts of niacin, an increase in excretion of niacin metabolites occurred at the level of 8 to 10 mg. They suggest that niacin stores are adequate at this intake. It is interesting that niacin deficiency is

rare in Central America, where corn constitutes 80 per cent of the diet. This may be attributable to lime treatment of the corn and the use of beans (193).

Frazier and co-workers (194) studied the excretion of niacin metabolites in six healthy college women on four levels of niacin intake. When the diet contained 7.2 mg. of niacin, the subjects excreted more than 100 per cent as metabolites. Excretion balanced an intake of 11.3 mg. An intake of 14 to 16 mg. of niacin daily was recommended to provide a margin of safety in the dietary requirements of this age group.

The development of symptoms of pellagra in an English child on a restricted diet (195) is described.

Pantothenic acid.—Bean and his associates (196, 197) have reported in detail the results of the daily administration for 25 days of 0.5 gm. of a pantothenic acid analogue, ω-methylpantothenic acid, to four volunteer subjects. At this time the deficiency symptoms became pronounced and included neuromotor disorders, cardiovascular instability, and complaints referable to the digestive tract. There were biochemical alterations in systems known to be mediated by pantothenic acid in animal tissue, such as changes in acetylation, blood cholesterol, and steroid hormone excretion. When 4 gm. of pantothenic acid daily were added to the diet for six days, recovery did not occur. The experimental diet was therefore discontinued and cortisone administered. Although absolute proof of a pantothenic acid deficiency state could not be obtained through the reversal of symptoms by addition of the vitamin itself, it is possible that the analogue acts by blocking the conversion of the vitamin to an active metabolic form, as appears to be the case with some folic acid antimetabolites. In this situation administration of pantothenic acid would be relatively ineffective. In a further report (198) it was found that two of three subjects receiving ω-methylpantothenic acid experienced a severe depression of gastric secretion and motility which returned to normal when the experiment was discontinued.

Vitamin D.—There are further reports (199) of excessive vitamin D intake causing hypercalcemia and renal lesions in infants and children. Creery & Neill (200) surveyed the dietary intake of 1087 Belfast infants and found 15 per cent had vitamin D intakes below the recommended allowance of 700 I.U. Over 50 per cent of the infants received more than the recommended allowances, and these were infants on vitamin D fortified dried milks.

A very interesting development in vitamin D therapy is the use of A.T.10, dihydrotachysterol, in alleviating symptoms in postoperative tetany (201) and rickets (202) after patients became resistant to vitamin D. Jonxis (203) reported that the urinary excretion of some amino acids is increased in rickets and postulates that vitamin D has an effect on amino acid reabsorption.

Other vitamins.—An anti-thiamine effect of a naturally occurring substance in bracken ferns has been reported (204). In seven out of eight sub-

jects, the urinary excretion of thiamine decreased when raw bracken fern fronds were added to a standard basal diet. Since the bracken fern is used as food in many countries, further study of its possible effect on thiamine nutrition is desirable. Sinclair (205) has suggested that alcoholic polyneuropathy which appears to be closely related to thiamine deficiency may be caused not by a dietary deficiency per se but by blocking of the metabolic action of thiamine through decreased phosphorylation or interference with the formation of lipoic acid. Holt & Snyderman (206) measured thiamine excretion in two infants and found less excretion on a high carbohydrate than on a high fat diet, presumably because more is required for metabolic purposes on the high carbohydrate diet.

Smith and co-workers (207) have studied thiamine allowances for adolescent boys and suggest a value of 1.36 ± 0.047 mg. per day or 0.38 mg. per 1000 calories, an amount that is in line with published thiamine requirements for adults. De, Datta & Mankad (208) using the 10 mg. saturation test concluded that Indian diets containing 0.83 to 1.35 mg. of riboflavin per day were adequate for this vitamin.

Potgieter and others (209) studied serum levels and urinary excretion of ascorbic acid in 20 women on varying but controlled intakes of the vitamin. At a 45 mg. intake, the serum level fell from 0.97 to 0.13 mg. per 100 ml. in five months. The maximum serum level of 1.49 mg. was reached after supplementation with 50 mg. Eight-hour night urinary output did not rise above the average value of 5 mg. until a 100 mg. daily supplement was added; then it increased to 30 mg. The question the investigators pose is what degree of tissue saturation is necessary for optimal ascorbic acid nutriture.

Caster & Mickelsen (210) conclude from a review that the serum vitamin A level is an unreliable measure of vitamin A nutrition and serum carotene is a better value. It has been found that increasing the intake of pyridoxine results in an increase in the blood level of transaminase (211). Severinghaus (212) reports that large doses of vitamin K given to premature infants have resulted in the development of hemolytic anemia.

Crump & Tully (213) have studied the effect of vitamin supplements given to children with a diagnosis of malnutrition and anorexia as a result of chronic illness and have found a general improvement in health in many instances. Ferguson (214) administered methionine and vitamins of the B-complex to 130 pregnant women and found no effect on the women or on mature babies, but the weight of premature babies was increased. The state of present knowledge in regard to vitamin requirements was reviewed by Pett (215).

Nutritional deficiencies induced by the action of drugs given for various therapeutic purposes may become a serious problem. A case in point is the development of a pyridoxine deficiency during administration of isonicotinic hydrazide to tuberculous patients (216). For this reason, the symposium held on antimetabolites (217) with discussion of modes of action and

therapeutic implications is of particular interest since many of the compounds discussed are analogues of essential nutrients.

FOOD ADDITIVES AND DIETETIC FOODS

Food additives include (*a*) those chemicals added deliberately to commonly used foodstuffs to prevent subnormal nutrition and frank deficiency disease, as the addition of iodide to table salt and of vitamin D to milk, (*b*) those resulting from the contamination of processed and packaged foodstuffs with machine lubricants, metals, and various chemicals used in protective wrappers and containers, (*c*) those present in agricultural products as the result of the use of chemicals in the fertilization of soils and in the control of pests, both before and after the harvesting of crops, and (*d*) those agents employed to increase storage life with a minimum loss of consumer acceptability and nutritional quality. The importance of this problem with its many ramifications is attested by the formation in 1950 and by the subsequent accomplishments of the Food Protection Committee of the National Research Council's Food and Nutrition Board. The Committee with the active assistance of a liaison panel of representatives of industry, trade associations, federal control and research agencies, and scientific and technical societies has evaluated critically the available information on the use of chemical additives and has stimulated the development of methods of analysis and the carrying out of basic studies on additives of questionable safety. The seventh and most recent publication of the Food Protection Committee deals with the safety of the artificial food sweeteners, saccharin, and the cyclamates (218). In addition to the earlier reports of this committee, techniques and procedures useful in the determination of the suitability of food additives have been reviewed by Deuel (219) and by Maynard (220).

The use of artificial, nonnutritive sweeteners has grown rapidly in recent years, largely because of the public interest in weight-reducing regimens and because of vain hope that herein was a solution to the problem of obesity. At the request of the Food and Drug Administration, the Food and Nutrition Board has considered the nutritional and public health aspects of the widespread consumption of artificially sweetened foods as well as the question of the potential hazard of a large scale consumption of these food additives. The conclusions of the Board on artificial sweeteners have been published as a policy statement (221). The report emphasized the fact that foodstuffs sweetened with saccharin or cyclamates have no favorable influence *per se* on diabetics or on those following weight-reducing programs except as the foods in question are used in feeding regimens in which there is control of the character or quality of the total food intake. Restriction of the use of the artificial sweeteners to properly labeled and controlled "special purpose" foods was recommended.

Numerous proposals to extend the enrichment program have been made. Foremost among these is the supplementation of prepared animal foods,

consisting in large part of grains, with amino acids, notably with lysine, methionine, threonine, and tryptophan (222). This would appear to be an economic problem depending on greater gains in weight per pound of enriched food compared with the cost of the supplementation. Enrichment of white flour with the limiting amino acid, lysine, may be justified for use in human dietaries in areas in which wheat or corn is the primary source of protein or, more generally, in periods of extreme shortage of food. It is believed a sound nutritional policy to avoid enrichment of foods for human use in the absence of evidence of a deficiency of the nutrient in question. Enrichment is not a satisfactory substitute procedure if optimum nutrition can be realized by education in the proper choice and preparation of foods and by improvement of processing methods.

The whole problem of the provision of a balanced mixture of amino acids in diets needs further study. In particular, more extensive analytical data on the amino acid content of common foodstuffs should be available to permit the efficient use of supplementary proteins. The role of other nutrients, such as vitamin B_{12} needs clarification, with special reference to differences between animal and plant proteins, before extensive supplementation with amino acids is attempted.

Antibiotics are now in the category of chemical additives and are currently added to certain animal foods. Although some of the effects of antibiotics in animal nutrition are fairly well defined, their potential effects on human nutrition are almost completely unknown. Stokstad (223) has reviewed the work with animals and concludes that the nutritive effects of these drugs vary with the types of microflora present in the gastrointestinal tract. A similar generalization might well apply in human nutrition. Haight & Pierce (224) studied the administration of chlortetracycline (aureomycin) or penicillin in 330 Navy recruits averaging 18.5 years of age and, after a seven-week period, found that the recruits receiving the antibiotics had gained more weight than a control group receiving placebos. Moustafa Kamal Badr El Din (225) observed that the administration of chlortetracycline in 11 Egyptian babies with infantile wasting resulted in a very significant increase in weight over that of infants in control groups who received dietary management and repeated blood transfusions. Chlortetracycline had a consistently stimulating effect on rate of gain in height and weight in Central American children. It is probably significant that oral administration of the antibiotics was found effective only during the season when diarrhea and intestinal disorders were most prevalent (38). There is also a report (226) that chlortetracycline increased the absorption of vitamin B_{12} in the blind intestinal loop syndrome. The need for controlled studies on the nutritional effects of long continued administration of antibiotics is clearly indicated.

The manufacture of so-called "dietetic foods" has become an important industry. Among these are artificially sweetened foods for those who must restrict their intake of sugar, products prepared without added salt for those who should restrict their intake of sodium, and special foods for use by al-

lergic subjects. Interestingly, a low-sodium milk is now on the market. The reviewers have been unable to find a complete report showing the change in the composition of the milk after the ion exchange treatment. The importance of the problem justifies such an analysis, because the treated milk may be used in highly restricted feeding programs.

SURVEYS AND SPECIAL STUDIES

Various symposia and reviews have considered nutrition in relation to specific aspects of health or environment. Silberberg & Silberberg (227) have reviewed the evidence in both animals and humans for the effect of the nutritional state on the life span. Moderate dietary restriction during certain periods of life increases longevity in some species, at least, but exact age limits have not been determined. The nutrition of blood cell formation was the topic of a recent symposium (228), and studies relating to nutritional hepatic injury have been reviewed (229). Brozek (230) reported the results of an investigation under controlled laboratory conditions of the effect of diet on behavior, particularly in regard to work. Nutrition as a military problem (231), its evaluation (63, 232) and its relation to climatic stress (233) have also received consideration. Kraybill (234) has discussed the effects of radiation sterilization on the nutritive value of foods, with special reference to military rations. Degradative changes in vitamins, fats, and proteins occur and attempts are being made to find improved procedures which will yield acceptable and safe products.

Several experimental studies on the nutritional status of various age groups have been completed during the past year. To learn more about the food habits of aging people and to determine the relation of food habits to physical health, Gillum and co-workers (235 to 240) selected 577 physically and mentally well men and women over 50 years of age and obtained dietary histories, medical examinations, and laboratory tests for hemoglobin, blood glucose, serum ascorbic acid, serum vitamin A and carotene, serum cholesterol, serum proteins, and other nitrogenous constituents of blood. Some of the findings are as follows: hemoglobin levels but not total serum protein were directly affected by the dietary protein and iron; serum ascorbic acid levels and the health of the teeth and gums were positively correlated with the ascorbic acid of the diet; males appeared to require a greater ascorbic acid intake than females to maintain equal serum levels; serum cholesterol levels were significantly higher in women than in men and in both were correlated positively with dietary cholesterol and fat; and no relationship between blood pressure and serum cholesterol levels was noted. The utilization of calcium, phosphorus, and nitrogen in older people on controlled diets has been reported (241, 242). In one study (243) the effect of androgen therapy was included.

The nutrition of mothers and infants has been studied in this country (244, 245, 246) and in Australia (247). It is reported (248) that the incidence of prematurity increases with a decrease in nutritional status and that high

caloric intakes predispose to preeclamptic toxemia (249). Dietary surveys of school children were made in Iowa (250, 251, 252), in Idaho (253, 254), in Rhode Island (255), and in Connecticut (256), as well as on the Gold Coast of Africa (257, 258). Many of these surveys have included blood levels of various nutrients for the purpose of establishing ranges for normal children. Other studies included measurement of blood levels of carotene and of caloric intakes in a group of preadolescent girls (259, 260), the response of boys 12 to 14 years old to a series of different breakfasts (261), and the effect of various supplementations in the diets of undernourished children in India (262, 263, 264).

Widdowson & McCance (265) have compared the nutritive value of bread made from flours of different extraction rates. The subjects were children from two German orphanages who had been receiving limited rations containing about 10 gm. of animal protein per day and who were undernourished by British standards. They received in addition to their regular ration as much bread as they wanted, prepared from flours of varying degrees of extraction. Some of the flours were enriched. All the children experienced a remarkable increase in growth rate regardless of the flour used. Milk supplements gave no further increase. This interesting study with its evidence of apparent adequacy for growth and development of a protein mixture containing very small amounts of animal protein provides much information of value to nutritionists and raises some pertinent questions.

The composition of the diet in various parts of the world has received consideration. There are two reports from Great Britain: a survey of family diet and health in pre-war Britain (266) and a food survey completed in 1954 (267). The diet in several regions of Africa (268, 269, 270), Malaya (271), and India (272 to 277) has been evaluated. The diet of the lower income classes in Finland has been found to be low in vitamin A and ascorbic acid (278). Estimates of the iron, calcium, and phosphorus intake of the Japanese have been reported (279, 280, 281).

Attention is again called to extensive surveys and to the accompanying field and laboratory research on nutritional problems in Central America (38). The Joint Food and Agriculture Organization, World Health Organization Expert Committee on Nutrition (282) has emphasized the importance of the proper educational instruction of populations which may receive food supplements and the necessity of relating the instruction realistically to the dietary habits of each population group.

The relation of the nutritive value of agricultural products to soil fertility has been studied in a series of experiments conducted over a ten-year period at Michigan State University (283). Crops produced by fertilized and by depleted soils did not differ appreciably in nutritive value. Dairy cattle maintained on these foodstuffs showed no differences in health, reproduction, or milk production and composition that could be related to the source of the rations. Likewise, the nutritive value of the milk, as tested by rat growth experiments, was not affected by soil fertility.

Various physiologic and pathologic symptoms believed to be the results of malnutrition are the subject of some interesting studies. A moderate anemia that is widespread among the Alaskan Eskimos was associated with a diet of poor quality (284). Likewise anemia and infection in Pakistan were rampant among low income groups (285). Pancreatic dysfunction occurred frequently in undernourished Jamaican children (286). Zubiran and others (287) have studied endocrine disturbances among the undernourished and suggest that lowered utilization of hormones by malnourished tissues results in an increased concentration in the blood of sufficient magnitude to inhibit the pituitary gland. Studies on 22 Americans (288) who had been prisoners of war in Korea indicated that their irreversible amblyopia was probably the result of malnutrition. A nutritional spinal ataxia (289) and a neurologic syndrome (290) characterized by tremors and rigidity of the extremities were associated with multiple deficient diets. Nutritional edema was present among migrant workers in Cape Town who lived on a diet of maize (291). Instances of enlargement of the parotid gland (292, 293) occurring during nutritional rehabilitation were attributed to changes in the fluid composition of the body tissues.

In the management of disease by diet therapy, a study has been reported of 71 juvenile diabetics who were evaluated as to diet, particularly caloric requirements, and to growth over a seven-year period (294). Hayes (295) reported that the "dumping syndrome" associated with postgastrectomy operations is caused by the presence of hypermolar solutions in the jejunum and advocated the use of a high fat, high protein diet which hydrolyzes slowly.

Trulson (296) has continued to assess dietary study methods. A reference diet for human nutrition studies has been developed (297) which is acceptable to subjects for a month or longer.

A symposium was held by the British Nutrition Society (298) on alcohol and its nutritional significance. Verzar (299) has discussed the study of the United Nations Commission on the coca leaf. There is an apparent relationship between nutrition and use of the coca leaf and it is suggested that better nutrition may be the clue to the elimination of this dangerous habit.

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BIOCHEMISTRY OF CANCER[1]

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During the past year researches on the biochemistry of cancer have continued at an increasing pace, which places the reviewer at a correspondingly increased disadvantage. In treading a tortuous path between the opposing goals of all-inclusiveness and critical evaluation, the reviewer finds himself entangled in a maze of almost inexhaustive literature (over 1200 references were considered), which taxes to the utmost his resources of judgment and common sense. A brilliant precedent has been set by Haddow (1), whose review on the same subject in last year's volume shows an impressive grasp of the fields of oncology, biology, physics, and chemistry and which is written in a characteristically lucid and elegant style.

Because of the stringent limitations of space, it has been necessary to curtail subject matter strictly to studies concerned directly with cancer. The following topics, many with enough material to justify separate reviews, have been arbitrarily excluded: effects of tumors on serum and urinary enzyme levels, diagnostic tests, clinical evaluation, the mechanisms of radiation effects, histochemistry, morphology, chemical syntheses and isolations, genetics, biology of transplantation, steroid metabolism, oncolytic viruses, plant and reptilian tumors, and cytogenetics. Lest the reader feel that this review is all-exclusive it should now be mentioned that carcinogenesis, tumor biochemistry and immunology, and chemotherapy will be dealt with in that order.

An attempt has been made to cover the literature through September, 1955, and there has been little duplication of references cited by Haddow (1).

Carcinogenesis

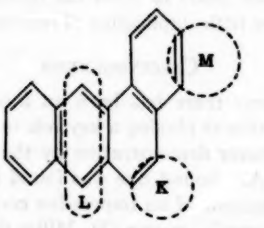
During the past year there has been an increased acceptance of the concept of protein deletion as playing a key role in the initiation of chemical carcinogenesis. The pioneer demonstration by the Millers in 1947 (2) that, following feeding of DAB, bound dye was found in the liver proteins, was followed by the development of an impressive correlation between protein-binding and the carcinogenic process (3). Miller demonstrated (4) that following application of BP to the skin of mice, fluorescence was liberated by

[1] The following abbreviations are used in this chapter: AAF for 2-acetylaminofluorene; ACTH for adrenocorticotrophic hormone; ADP for adenosinediphosphate; ATP for adenosinetriphosphate; BA for 1,2-benzanthracene; BP for benzpyrene; DAB for *p*-dimethylaminoazobenzene; DBA for dibenzanthracene; DMBA for 9,10-dimethyl-1,2-benzanthracene; DNA for deoxyribonucleic acid; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); MCA for methylcholanthrene; RNA for ribonucleic acid; TEM for triethylenemelamine.

hydrolysis of the proteins, and Wiest & Heidelberger (5), using labeled DBA, confirmed and extended this observation quantitatively. The phenomenon of protein-binding at the site of tumor formation has also been observed in the case of AAF (6). The fact that three chemically and carcinogenically different classes of compounds exhibit this phenomenon, suggests that it may reflect a mechanism common to chemical carcinogens, and which might extend to physical and viral carcinogenesis as well. In essence, the deletion theory, first proposed by Potter in 1944 (7), postulates the initial inactivation of an enzyme or enzyme system essential for the control of growth as a result of its combination with the carcinogen, and that subsequently, following cell division, some cells without this control mechanism would be formed; these would be cancer cells. This concept has been discussed by Rusch (8) from the point of view of differentiation and specialization. A similar view, somewhat different in emphasis, is presented by Rondoni (9).

The English School, as exemplified by Boyland (10), formerly considered that carcinogens produced genetic mutations by direct interaction with nucleic acids. However, a lack of correlation between mutagenicity and carcinogenicity has been pointed out by Burdette (11), and recently Haddow (1) has accepted the protein deletion hypothesis originated by the Wisconsin group. A mathematical model to test the mutation theory of carcinogenesis is proposed by Wollman (12), but no suitable experimental data are yet available.

A considerable advance has been made recently by the Pullmans (13, 14, 15) in the correlation between electronic structure of hydrocarbons and carcinogenic activity. They have calculated the pi electron distribution



of 37 hydrocarbons, and found that for greatest carcinogenic potency there should be a high electron density at K and a low density at L. They envisage the interaction of the carcinogen with tissues at the K region through quinonoid bonds, which would facilitate metabolic hydroxylation at M. Correlations between carcinogenic activity and fluorescence spectral shifts (16), coordination with silver ions (17), and "frontier electron" distribution (18) of hydrocarbons have been discussed.

Table I summarizes new reports of carcinogenic activities of well-defined, chemical compounds, exclusive of metals and plastics.

TABLE I

COMPOUNDS WITH CARCINOGENIC ACTIVITY

Compound	Tumor	Evaluation	Ref.
6(β-Hydroperoxy-Δ^4-cholestene-3-one	Fibrosarcomas	++	19
6-Hydroxy-Δ^4-cholestene-3,6-dione	Fibrosarcomas	+	20
Δ^4-Cholestene-3,6-dione	Fibrosarcomas	++	20
Cholesterol α-oxide	Fibrosarcomas	++	20
Thioacetamide	Bile Duct	+	21
Thiourea	Face	+	22
3,2'-Dimethyl-4-aminobiphenyl	Bladder	++	23
4-Fluoro-4-aminobiphenyl	Liver, Kidney, Intestine	++	24
4'-Fluoro-4-acetylaminobiphenyl	Liver, Mammary glands, Kidneys	++	25
4-Aminobiphenyl	Bladder	++	26
Monocrotaline	Liver	+	27
Retrorsine	Liver	+	28
Isatidine	Liver	+	28
Light Green SF	Spindle-cell sarcomas	+	29
1-(2-Tolylazo)-2-naphthol (oil orange TX)	Spindle-cell sarcomas, Intestine	+	30
Many new derivatives of 2-acetylaminofluorene, including fluoro substituted	Many types	++	31
4-Methylpyridine-2-azo-*p*-dimethylaniline and many derivatives	Liver	++	32
4'-Ethyl-4-methylaminoazobenzene and derivatives	Liver	++	33
Urethan	Skin	+	34
Triethylenemelamine	Skin	+	35
β-Propiolactone	Skin	++	35
4',9,10-Trimethyl-1,2,-benzanthracene	Skin, Sarcomas	+	36
6,7,9,10-Tetramethyl,1,2-benzanthracene	Skin, Sarcomas	+	36
Stearoylethyleneimine and derivatives	Skin, Sarcomas	++	37
Phenazine	Bladder	++	38
1,2,3,4-Dibenzophenazine	Bladder	++	38
1,2,5,6-Dibenzophenazine	Bladder	++	38
Phenol	Skin	+	39
p-Halogen phenols	Skin	+	39

Hydrocarbons.—Berenblum & Haran (40) have reconfirmed the fact that papillomas are produced by a single treatment of DMBA followed by 40 applications of croton oil, whereas none were produced with the reverse treatment. This was taken as further evidence in favor of separate initiating and promoting stages of carcinogenesis. Berenblum & Nechama (41, 42) have studied the factors involved in hydrocarbon induction of tumors of the forestomach. An intriguing observation of Graffi *et al.* (43) was the production of skin tumors at the site of croton oil application following intraperitoneal or peroral administration of DMBA. Klein has studied the time intervals that are optimal for croton oil promotion (44).

The invasion of wounded-grafted epidermis into the dermis and the subsequent appearance of "elastically degenerated" collagen, reported by Gillman *et al.* (45), together with the elegant skingraft studies by Marchant & Orr (46) with carcinogen-treated epidermis, and the further evidence for the importance of hair follicles in skin carcinogenesis adduced by Liang & Cowdrey (47), call attention to the essentiality of, and the participation of, the dermis in the carcinogenic process.

The induction of ovarian tumors by DMBA (48) has been reported. Jull (49) has found an increased incidence of mammary tumors in mice treated with progesterone following application of MCA to the skin. In thymectomized gonadectomized DBA/2 mice, treated similarly with the carcinogen, 100 per cent incidence of leukemia was observed by Kirschbaum & Liebelt (50), who also obtained thymic tumors following oral ingestion of MCA. Agate *et al.* (51) found that hypophysectomized animals, injected subcutaneously with BP, developed tumors, and Moon & Simpson (52) have shown that similar animals show a delay in MCA carcinogenesis. They reported a much lower tumor incidence in such mice, but their published curves imply that a higher incidence would have been obtained had the experiment been continued for a longer time. Thus it appears that the pituitary is not essential for the chemical production of sarcomas, but its absence probably affects the metabolism of the animal so as to slow down the carcinogenetic process.

A number of substances have been shown to affect the course of hydrocarbon carcinogenesis. The following compounds applied to the skin inhibit tumor formation: benzoquinone (53), hydroxylamine, sodium azide, and sodium nitrite (54), cortisone (55), and squalene (56), which chemically alters hydrocarbons (57). Cortisone given systemically increases the incidence of hydrocarbon tumors (58) as does an emulsion of unsaturated fatty acids (59) and Janus Green (60). Steiner (61) has found additive effects, inhibitions, and no effects, with subcutaneous mixtures of hydrocarbons of varying potency, thus emphasizing that not enough is known to warrant generalizations about these phenomena.

An elaborate statistical analysis of the induction of lung tumors by hydrocarbons and other substances has been provided by Shimkin & Polissar (62, 63).

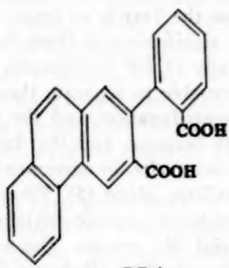
A considerable amount of work has centered about the metabolism of naphthalene, which, although noncarcinogenic, might be considered as a simpler prototype of the more complicated carcinogenic hydrocarbons. Boyland & Solomon (64) have isolated from the urine of rats and rabbits given naphthalene a compound which they have characterized as 1,2-dihydro-1-naphthyl glucosido uronic acid, and which yields the parent hydrocarbon on acid treatment. Administration of 1,2-dihydronaphthalene resulted in little urinary excretion of the 1,2-dihydrodiol, which suggests that the latter compound is formed by a perhydroxylation mechanism. The same uronic acid, together with 1-naphthyl sulfuric acid, and 2-naphthol,

has been isolated by Corner & Young (65) from the urine of rats dosed with the *trans*-1,2-dihydronaphthalene-1,2-diol; the authors have concisely summarized the metabolism of napthalene and its diol.

Berenblum & Schoental (66) have called attention to the fact that the structures (derivatives of 8,9-dihydrobenzpyrene-diol) postulated for the BPX and BPF metabolites of BP by the late Drs. Weigert & Mottram (67) have never been proved and are probably incorrect. Calcutt & Payne (68, 69, 70) have injected BP intraperitoneally into mice and determined the fluorescence in the nuclei, mitochondria, microsomes, and supernatant fractions of the livers, and by previously described procedures they have analyzed for BP and the above mentioned BPX and BPF derivatives. They found qualitatively that these substances occurred in all fractions in the free state, and in sub-fractions of the supernatant fluid, with no demonstration, however, that they were associated with proteins. Similar experiments were carried out in rats. Because the liver is an organ that is resistant to hydrocarbon carcinogenesis, the significance of these findings is difficult to evaluate. A more pertinent study of BP distribution has been carried out by Fiala *et al.* (71). They were able to separate the cell components of mouse epidermis by differential centrifugation, and the precipitated proteins were hydrolyzed, extracted with benzene, and the fluorescence measured. Very little BP was detected in the nuclei, in agreement with the protein-binding studies of DBA in submaxillary gland (5). Pihar (72) has found that suspensions of BP inhibit beef-heart succinic dehydrogenase and suggests that BP may inactivate essential SH groups. The weak absorption of BP to serum proteins has been described by Chalmers (73).

Graffi's group (74) have carried out extensive studies on the tissue distribution of fluorescence following skin application of BP, but no information as to the chemical nature of the fluorescent products is available. On the other hand, Moodie *et al.* (75) studied the fluorescence in protein hydrolysates of skin, following local application of several hydrocarbons, and found appreciable protein binding with the carcinogenic BP, DMBA, and DBA, but very little with the noncarcinogenic BA, anthracene, phenanthrene, and dibenzofluorene. By contrast, Woodhouse (76) reported approximately equal amounts of protein-bound fluorescence following skin treatment with BP and the noncarcinogenic 2′,6-DMBA and concluded that there was no relation between the processes of protein binding and carcinogenesis. However, because of uncertainties in fluorescence quantitation and tissue extraction, quantitative comparisons of different compounds based on fluorescence can be extremely erroneous; accurate determinations of radioactivity in skin proteins following applications of labeled BP and 2′6-DMBA showed that the latter was bound only one fourth as much as the former (77). In studies under comparable conditions of the binding to skin proteins of labeled phenanthrene, BA, 1,2,5,6-DBA, 1,2,3,4-DBA, DMBA, MCA, BP, and 2′6-DMBA, there was a perfect correlation between carcinogenic activity and the quantity of bound radioactivity, with

the exception of the noncarcinogenic 1,2,3,4-DBA, which was extensively bound (77). Darchun & Hadler (78) have also determined the quantity of protein-bound radioactivity following the application of labeled DMBA. Thus it appears that the bulk of the evidence favors the correlation of protein-binding with carcinogenic power and suggests that protein-binding is a necessary, if not sufficient, prerequisite for carcinogenesis. By means of carrier technique, Bhargava *et al.* (79) have shown that about 30 per cent of the protein-bound radioactivity is accounted for as 2-phenylphenanthrene-3,2′-dicarboxylic acid (PDA). It is of interest that this substance, which has now been shown to be bound through the carboxyl groups, represents attachment to the tissue protein at the "K" region of Pullman (15), but it remains for the future to determine whether this particular bound metabolite of DBA represents the actual carcinogenic complex.



PDA

Azo dyes.—The entire subject of hepatic tumors has been thoroughly reviewed in a supplement to the *Journal of the National Cancer Institute,* including a contribution by the Millers (80) which describes their important research in this field. The aminoazo dyes are metabolized by reduction of the azo linkage, by N-demethylation, by hydroxylation, and by binding to liver protein. In correlating structure with activity the inactivity of the 2,6-difluoro DAB, in contrast to the enhanced carcinogenicity of other fluorine-substituted compounds, suggests that a free 2-position is essential for biological activity. Since it had been suggested that the azo dyes are carcinogenic because of rearrangement to active benzidine or semidine products, several of these have been synthesized and shown to be noncarcinogenic.

Several compounds when administered to rats inhibit the carcinogenicity of azo dyes. These include *p*-hydroxypropiophenone (81), Trypan Blue (82), noncarcinogenic azo compounds (83), and 2,5-dimethylbenzimidazole (84). Punch biopsies of the liver were shown by Richardson (85) to speed up the induction of tumors by azo dyes, and Shelton (86) found that stilbesterol increased tumor incidence. Griffin, Richardson, and their group have shown that hypophysectomy prevents the production of liver tumors by azo dyes in the normal induction period, but that in such animals carcinogenesis

was restored by administration of ACTH and somatotrophin (87, 88). However, Li[3] has obtained tumors in hypophysectomized rats after more prolonged dye feeding, so that just as in the case of the carcinogenic hydrocarbons, the pituitary is not necessary for carcinogenesis, but doubtless its lack exerts a delaying action through indirect metabolic effects.

The Millers have shown that the N-methyl groups of the azo dyes are not capable of acting as a major source of the metabolically important labile methyl groups (89). White *et al.* (90) have studied the metabolism of N^{15}-labeled *p*-dimethylphenylenediamine, the split metabolite of DAB, in rats and have demonstrated extensive excretion and no formation of protein-bound material. Zischka *et al.* (91) measured the tissue distribution of C^{14} following administration of ring-labeled DAB and found no conversion to respiratory CO_2. The Millers and Rastogi (unpublished) have degraded the polar bound dye of DAB and have obtained dimethylamine. This shows that the dye is not bound through the N-methyl groups as previously supposed (3), and, taken together with the noncarcinogenicity of the 2,6-difluoro compound, it now seems likely that the dye is bound to protein through the 2-position.

2-Acetylaminofluorene and derivatives.—A broad review of AAF carcinogenesis from the structural, biological, and metabolic viewpoint has been written by Morris (92). In a very extensive series of compounds Miller *et al.* (31) have studied the structural requirements for activity in this series. They found that the fluorene nucleus was necessary for production of liver tumors and that a biphenyl ring system, with and without various 2,2′-bridges, is required for mammary tumor production. 2-Acetylaminophenanthrene showed some leukemogenic activity and produced a characteristic paralysis of the hind limbs.

Mori (93) has observed that prolonged feeding of liver powder markedly inhibited hepatoma production by AAF and completely suppressed mammary tumor induction. An opposite effect was observed by Morris *et al.* (94) who found that riboflavin added to the diet increased mammary and other tumors, while there was no effect on hepatoma induction. Wase (95), in experiments with labeled riboflavin, found that its biological half-life in liver was doubled by AAF feeding. AAF also induces tumors in fowls (96, 97), and Boyland *et al.* (98) produced bladder tumors in rats given AAF and supplementary tryptophan. A phenomenon similar to that with MCA (43) has been reported by Ritchie & Saffiotti (99), in which skin tumors appeared at the site of croton oil application following oral administration of AAF.

The urinary excretion of a large number of derivatives of AAF has been studied by Dyer (100), who found no correlation between the rate of excretion and carcinogenic activity. Weisburger *et al.* (101) observed that riboflavin deficiency delayed the absorption of labeled AAF, produced an in-

[3] Li, C. H. (Personal communication).

creased quantity of water-soluble metabolites, and had no effect upon the binding to liver protein. E. K. Weisburger (102) demonstrated that 3-AAF, a weakly carcinogenic isomer, is metabolically hydroxylated in the 2-position. Gutmann & Peters (103) have shown that AAF is deacetylated and hydroxylated in the 7-position by liver slices. J. H. Weisburger observed a similar deacetylation in liver slices, homogenates, and acetone powder extracts (104). There was no evidence of deacetylation in intestinal segments but deacetylation in liver of various positional isomers of AAF of varying carcinogenicities was observed. Protein-bound C^{14} was obtained in homogenates of liver, but not of hepatoma, with the majority in the supernatant fraction. Peters & Gutmann (105) have demonstrated the acetylation of 2-aminofluorene in liver slices with acetyl coenzyme A as the probable agent, as well as protein binding.

Other compounds.—The metabolism in rats of labeled β-naphthylamine, the potent bladder carcinogen, has been investigated by Henson *et al.* (106). They found, following intraperitoneal injection, a rapid absorption, rapid urinary excretion, no selective localization in the bladder, and a very lengthy bodily retention of a small amount of radioactivity. The fate of β-naphthylamine in liver and kidney slices has been studied by Booth *et al.* (107), who have isolated 2-amino-1-, 2-amino-6-, and 2-acetamido-6-naphthyl sulfuric acids. The latter compound is hydrolyzed by kidney slices to 2-acetamido-6-naphthol.

It has been amply demonstrated by Roe & Salaman (34, 35) that urethan applied to the skin is capable of acting as an initiator of carcinogenesis provided that croton oil is applied subsequently. Firth & Roe (108) have failed to detect an infective etiology in the production of lung tumors with this agent, and Rogers (109) believes that urethan is converted metabolically into the active carcinogen.

The Silberbergs (110) have demonstrated that ACTH is leukemogenic in mice. Several fibrosarcomas of the buttocks have been described (111) in patients that had received intramuscular penicillin in sesame oil, and Boutwell *et al.* (39) have demonstrated carcinogenic activity of several phenols. Paschkis *et al.* (112) have reviewed their provocative work on the induction of neoplasms by the injection of tumor chromatin, and Saffiotti and Shubik (113) reported the initiation of skin tumors by burning, followed by croton oil.

The baffling problem of carcinogenesis by implanted plastics has received considerable attention. The Oppenheimers and co-workers (114) have induced malignant tumors with the following films: Cellophane, Dacron, polyethylene, polyvinyl chloride, Silastic, Pliofilm, Nylon, polymethyl methacrylate, polystyrene, Saran, Ivalon, Kel-F, Teflon, and silk. Their findings with polyethylene have been confirmed by Druckrey & Schmähl (115) and by Bering *et al.* (116); their observations on polymethyl methacrylate have been confirmed by Laskin *et al.* (117) and Nothdurft (118). The latter found that plastic powder, balls, rods, bristles, and films with holes

in them are not as carcinogenic as intact films; he has also found films of ivory, gold, silver, and platinum to be carcinogenic (119). In view of the wide variety of chemical properties of these substances and of the fact that C^{14} could not be detected in respiratory CO_2 and tissues following embedding of labeled polystyrene films, and that only a minute amount was found in the urine (114), the hypothesis has been advanced that "active centers" in the polymer might be produced metabolically, which could bind to protein and thus initiate cancer.

The pioneering work of Hueper and his group on metal carcinogenesis in rats has continued; nickel and uranium are carcinogenic, chromium very weakly so, and asbestos, beryllium, and arsenic are not carcinogenic in rats, although they appear to be in humans (120, 121, 122).

The sacrosanct status of croton oil as possessing only promoting activity has been shattered by three demonstrations of its carcinogenic activity (35, 39, 123), but the effect of this finding upon the theory of two stage carcinogenesis is not yet clear. Danneel & Weissenfels (124) have fractionated croton oil and have succeeded in separating the promoting activity from the irritating and hyperplastic fraction. Setälä *et al.* (125) have found marked promoting but no carcinogenic activity in the detergents, Span 20 and Tween 60.

Environmental cancer.—This subject has been reviewed in an exceptionally thorough and scholarly fashion by Hueper (126), in which the increasing carcinogenic character of the environment in our highly industrialized civilization points up the necessity for vigorous counteraction by industry and public health authorities.

Poel *et al.* (127) have demonstrated carcinogenic activity in coal tar distillates containing no BP, and Eby *et al.* (128) have reduced the carcinogenicity of high boiling petroleum fractions by ozonization. Steiner (129) has demonstrated the presence of BP in carbon blacks used in tire manufacturing; however, elution is required in order for carcinogenic activity to be elicited. The industrial smoke of Newcastle, England, and Diesel engine fumes have shown carcinogenic activity in the tests of Clemo *et al.* (130). Kotin & Falk (131) have produced tumors from a fraction isolated from Los Angeles "smog" that contains predominantly aliphatic unsaturated hydrocarbons. They have also shown that "smog" attacks aromatic hydrocarbons; the latter were not detectable at autopsy in the soot from the lungs of "average city dwellers" (132). Truhaut (133) and Druckrey (134) have reviewed the problem of carcinogenicity in food-coloring dyes.

The etiology of lung cancer in man is receiving increased attention. Gilliam (135) has analyzed the data on the mortality attributed to lung cancer in large cities of the United States; he was unable to correlate the data with the degree of industrialization or other factors. The statistics available on the relationship between cigarette smoking and lung cancer have been reviewed critically by Hammond (136), Cutler & Loveland (137), and Doll (138). There appears to be general agreement that there is a close

relationship, but statistical data alone cannot prove that the relationship is causal. As Doll (138) concludes,

> it is a reasonable presumption that the changes which have taken place in tobacco consumption (in amount and in method) are responsible for the major part of the real increase in mortality. . . . Two other factors have at times received considerable prominence, namely atmospheric pollution and hereditary susceptibility. The evidence concerning the former permits no definite conclusion, save only that it is not independently responsible for a large proportion of cases nor for the recent increase in mortality. There is no evidence concerning the latter, though doubtless susceptibility to inspired carcinogens varies as does susceptibility to other environmental stimuli.

Holsti & Ermala have produced carcinoma of the bladder after oral administration of tobacco tar (139) and have obtained a "striking correlation" between the clinical incidence of lung tumors and the anatomical localization of tobacco smoke in mice and in a full-scale plastic model of the human respiratory tract (140). Wynder *et al.* (141) have produced skin tumors from tobacco tar in several strains of mice and conclude,

> it can only be assumed that the carcinogen(s) isolated for the animal will also be the carcinogen active for man. The fact that in tobacco research the animal experiment is in line with the human experience emphasizes this possibility.

Substances with fluorescence spectra similar to BP have been found in cigarette tar (142, 143, 144), combustion products of cigarette paper (145), and snuff (146); however, the presence of BP has not yet been unequivocally proved. Fractionation studies indicate that other substances, carcinogenic to mice, are also present (145, 147). Druckrey and his group have shown that irradiation decreases the fluorescence intensity of cigarette smoke (148), and that light-excited, unstable substances are also found (149).

Hormones and radiation.—The subject of neoplasia and internal environment has been reviewed by Bielschowsky (150) in a paper in which he deals with the role of hormones in the induction and spontaneous regression of tumors. Morris (151) has summarized existing knowledge on the induction and metabolism of tumors of the thyroid gland and concludes that tumors not produced by irradiation arise as the result of a constant stimulation of the thyroid by thyrotropin evoked by a decreased circulation of thyroid hormone. Mühlbock (152) has discussed the factors concerned in the production of mammary tumors, which can be accomplished by the implantation of estrogen pellets (153). Cervical and vaginal cancer have been induced in mice by intravaginal applications of estrogens (154), and Gardner has also shown (155) that estrogens augment and testosterone inhibits x-radiation leukemogenesis. The tumors induced by the hormonal imbalance resulting from the transplantation of ovaries to the spleen have been studied histologically and endocrinologically by Gardner (156); some of these tumors secrete estrogens. Martinez & Bittner (157) have shown that removal of one adrenal gland of mice at the time of oophorectomy did not prevent the formation of adrenal tumors in the remaining gland. Furth and his col-

leagues (158) have obtained pituitary tumors in thyroidectomized animals, thus disproving the necessity of irradiation and permitting thyroidectomized hosts to carry transplants of dependent pituitary tumors. They have also shown that x-radiation increases, proportionally to the dose (159), the incidence of spontaneous pituitary tumors in female mice.

Kaplan and his group have been studying for some time the induction of lymphoid tumors and have concluded that the thymus is the primary site of such tumor formation (160). They have observed an inhibition of tumor development following injection of bone-marrow suspensions into irradiated mice (161). In studying the role of the pituitary in lymphoid tumors Nagareda & Kaplan (162) did not find an inhibition of radiation-induced tumors and of normal growth of transplants in hypophysectomized rats (163), another example of the nonessentiality of the pituitary for carcinogenesis. In an extremely provocative experiment Kaplan *et al.* (164) have shown that malignant lymphoid tumors are induced in nonirradiated homologous thymus glands implanted into thymectomized, previously irradiated mice, and have adduced evidence that the transplanted cells play the essential role in the genesis of the tumor. Thus it appears that a completely indirect mechanism of tumor induction obtains in this case, and Kaplan believes, since the tumor arose in a tissue not exposed to the carcinogenic agent, that it arises in the thymus as a result of the stimulus to regeneration caused by injury to the thymus and bone-marrow, and that there is no sudden irreversible change produced at the time of exposure to the leukemogenic agent. If Kaplan's interpretation of this experiment is correct, the currently accepted ideas about carcinogenesis may require revision.

Koletsky & Gustafson (165) have observed a large number and variety of tumors in rats that survived a single whole-body x-radiation, of 640 r, and Lorenz *et al.* (166) have recorded the shortened survival time and increased incidence of lymphosarcoma in mice which received daily small (0.11 r) irradiations. Pullinger has induced mammary carcinoma in mice without the milk factor by local irradiation (167), and Cloudman *et al.* (168) have observed additive effects of MCA treatment and β-ray irradiation of skin in the induction of tumors. When Rogers (169) exposed fetal mouse lung *in vitro* to a single exposure of ultraviolet light and transplanted the tissue into mice of the same strain, pulmonary adenomas were induced.

Carcinogenic viruses.—Bryan and his colleagues are carrying out an extensive study of the chemical and biological properties of the Rous sarcoma virus, and have shown that the precision of dose-response data in the virus field is comparable to that obtained in other biological systems (170). Partially purified preparations of this virus are stable in the frozen state (171); its density in sucrose is 1.150 (172), and its sedimentation constant is 655 S, corresponding to a diameter of 89 mμ (173). The authors have shown that the amount of extractable virus is related to the initiating dose, but that virus was not detectable in tumors induced by small quantities of virus, thus showing that it is unjustified to conclude that tumors are of

nonviral origin if virus can not be detected in tumor extracts (174). Harris, working with the same virus, has found an appreciable concentration of hyaluronic acid in fowl sarcoma tumor tissue (175), and has demonstrated acid and alkaline phosphatase activity in concentrates of the virus (176); however, the alkaline phosphatase was not associated with infective activity (177). Harris has also succeeded in growing the Rous virus in the chorioallantoic membrane of fertile eggs (178).

The Beards have been working on the purification of the avian erythromyeloblastic leukosis virus and have found that the natural immunity of the host to the virus increases with age (179). The pH-stability range for infectivity is narrow, and morphological changes were not observed in the pH-inactivated virus (180). The adenosinetriphosphatase activity that is inseparable from the virus is most probably an integral part of the virus molecule and behaves like a typical enzyme (181), which is further substantiated by kinetic analysis and inhibition by ADP (182).

The research on the induction of leukemias by cell-free filtrates of tumor cells has been extended during the past year. Although there is currently no agreement on leukemias,[3] it is now well established by Gross (183), Stewart (184), and Law *et al.* (185) that parotid gland tumors may be induced by extracts of leukemic cells and even of normal cells (183). Gross (186) has shown in a large series of animals that a 28 per cent incidence of leukemia was obtained in a Bittner substrain of C3H mice, whereas only a 4 per cent incidence was found with the National Cancer Institute C3H substrain following inoculation of filtered extracts of Ak leukemic cells. Under the same conditions, Stewart (187) obtained up to a 70 per cent incidence of leukemias in (C3Hf $\times$ AKR)F_1 hybrid mice. On the other hand, Law *et al.* (185) failed to obtain any significant incidence of leukemias, even in the same hybrids used by Stewart (187) and conclude that all previous experiments [Stewart (187) not mentioned] "are totally inadequate to establish a direct relationship between one or more cell-free agents and specific morphologic forms of neoplasm." However, Graffi and his colleagues and Schmidt have recorded a high incidence of leukemias following inoculation of young mice with centrifuged and filtered extracts of a number of transplanted tumors, including sarcoma 37, sarcoma 1, sarcoma 2, a DMBA-induced transplantable spindle-cell sarcoma, and as high as an 83 per cent incidence from the Ehrlich ascites carcinoma (188, 189, 190). Only in the latter case (190) are numerical or histological data reported. If these astonishing findings can be confirmed, an important new milestone in viral carcinogenesis will have been reached.

Biochemical changes during carcinogenesis.—The Japanese workers have been actively investigating various enzyme levels in the livers of rats undergoing carcinogenesis, and similar results have been observed with

[3] The results of Gross on leukemia production have been fully confirmed by Woolley & Small (*Proc. Am. Assoc. Cancer Research*, **2**, 158 (1956)

azo dyes and AAF. The following enzyme levels and reactions decreased steadily in the precancerous livers to a low value in the hepatoma: asparaginase (191), esterase (192), rhodanease (193), synthesis of *p*-aminohippuric acid (undetectable in tumor) (194), deamidase of halogenated fatty acids (195), catalase and uricase (196), and arginase (197). A change was not found in guanase (197), and the levels of choline oxidase (198) and fatty acid deamidase (199) remained constant in the preneoplastic livers and decreased markedly in the hepatoma. Alkaline phosphatase remained constant and increased in the tumor (192). Carruthers *et al.* (200) found polarographically that there was no change in the levels of pyridine nucleotides in livers undergoing azo dye carcinogenesis, but a marked decrease in the tumors. In tracer experiments Fish *et al.* (201) did not observe a change in liver cholesterol metabolism during carcinogenesis, but a decreased turnover in the hepatoma. Although it appears, in general, that various enzyme levels are lowered during liver carcinogenesis, and many previous examples are known, their metabolic roles are so divergent that to this reviewer no generalizations can be discerned.

Changes in nucleic acid metabolism have also been studied during the course of hepatic carcinogenesis. Belousova has found periodic alterations of RNA content and a shift from mitochondria to microsomes, while the DNA and proteins remained constant (202). Rodriguez *et al.* (203) have reported an increase in the DNA:potassium ratio during carcinogenesis. However, Ward & Griffin (204) did not observe a change in the incorporation of P^{32} into nuclear RNA in the preneoplastic livers. Rutman *et al.* (205) found an increasing amount of protein and RNA in the nucleus with a corresponding loss from the cytoplasmic fractions during carcinogenesis, and a large increase of DNA in the hepatoma. They have also demonstrated an increased utilization of uracil for RNA synthesis and of alanine for protein synthesis during carcinogenesis, with an elevated value in the hepatoma (205).

Kandutsch & Baumann (206) have found that the application of MCA to the skin of mice lowered the level of Δ^7-cholestenone in the skin towards that found in tumors; other strong carcinogens and croton oil were found to have a similar effect; noncarcinogenic hydrocarbons had no effect (207). The significance of these observations to the process of skin carcinogenesis is not clear.

Biochemistry of Tumors

A number of reviews of topics of oncological biochemical interest are found in the proceedings of the First Canadian Cancer Conference (208). Reviews of various aspects of cancer biochemistry have been written by Haddow (1), von Euler (209), Greenberg (210), Lang (211), Lemon *et al.* (212), Law (213), Schmidt (214), and Weinhouse (215).

Oxidative metabolism.—Jedeikin & Weinhouse (216) have determined the ratio of DPN to DPNH in a variety of normal and neoplastic tissues and

found that the ratio and quantities in tumors were intermediate among the normal tissues. Wenner & Weinhouse have studied the effects of dinitrophenol and fluoride on the oxidation of glucose in various normal and tumor tissues in order to evaluate the contribution of oxidative phosphorylation; they observed no significant differences (217). The effect of dinitrophenol on the oxidation of glucose in intact Ehrlich ascites cells is slight, probably because of low permeability, according to Schacter (218). By comparing in tumor slices the oxidation to CO_2 of glucoses, labeled in two different positions, Abraham *et al.* (219) and Emmelot *et al.* (220) concluded that tumors possess a pathway of oxidation in addition to the Embden-Meyerhof glycolysis and Krebs cycle. The latter (220) failed to obtain fatty acid oxidation in tumor mitochondria from KCl homogenates (221) but succeeded with sucrose homogenates, which they believe decreased the production of latent adenosinetriphosphatase (222). Allard has determined the levels of acid and alkaline phosphatase and ribonuclease in hepatoma mitochondria and concluded that their properties are different from those of other tissues (223). An inhibition of aerobic glycolysis and an increase of respiration in homogenates of ascites cells was obtained by Kertész & Albano (224) by the addition of a polyphenoloxidase terminal respiratory system. Schmidt & Schlief (225) found no lack of cytochrome-*c* in tumors. Neufeld *et al.* (226) have found a peroxidase in the mitochondria of the Walker tumor and suggest that it may participate in oxidations to circumvent the deficiency of cytochrome oxidase. Weinhouse (215) has discussed very thoroughly his work and that of others on the oxidative metabolism of neoplastic tissue studied *in vitro*; he concludes that the most characteristic property of tumors is not a deficient oxidation, but a high aerobic glycolysis, the explanation for which is not yet understood.

The results of Busch and his colleagues in studies *in vivo*, stand in sharp contrast to the investigations, *in vitro*, just cited. They found, following the injection of acetate-1-C^{14} to rats bearing several types of tumors, that although acetate is very rapidly metabolized, primarily to glutamate in most normal tissues, tumors *in situ* are virtually incapable of oxidizing this substance (227). Similar studies with labeled pyruvate showed a rapid conversion to amino acids in normal tissue, essentially no amination, and an almost complete conversion to lactic acid in the tumors (228). These results show that in the animal, tumors exhibit a very high anaerobic glycolysis and essentially no oxidation, and some studies, *in vitro*, of Strength & Seibert (229) on electron transport in tumors tend to bear this out. It is evident that there is a wide divergence of results bearing on oxidative metabolism when studied *in vitro* or in the living animal which cannot be attributed to experimental inaccuracies. Clearly, more work must be done to reconcile these differences, but it should be added, perhaps tritely, that valid experiments in intact animals must approximate more closely the true physiological state of tumors than do slices, homogenates, mitochondria, and isolated enzymes, and those working *in vitro* can no longer profitably ignore or discount the experiments done in intact animals.

Glycolysis.—Holzer *et al.* have confirmed the presence of most of the glycolytic enzymes in Ehrlich ascites tumors (230), but glucose-6-phosphatase or an inhibitor of it has not been found in hepatomas by Weber & Cantero (231). An elegant kinetic study of the rates of aerobic glycolysis in human normal and leukemic leucocytes has been carried out by Beck (232), who found differences in rates between normal and leukemic cells, suggestions that most enzymes were not operating at their full capacity, and that no single enzyme could be considered rate-limiting. A tremendous amount of data on the regulatory effects of insulin on glycolysis of a transplanted melanoma have been reported by Burk's group (233), but no clear-cut conclusions could be drawn by this reviewer. Sibley & Fleisher (234) have shown that in a number of human tumors the content of aldolase was higher than that of adjacent tissue, and Schlief & Schmidt (235) found no correlation between the growth rate of the ascites tumor and its aldolase and hexokinase activities. Groth & LePage (236) have demonstrated the production of propanediol phosphate from pyruvate in anaerobically glycolyzing tumor homogenates, and Emmelot & Bosch have confirmed many earlier observations (cf. 215) that tumor slices oxidize pyruvate to CO_2 more rapidly than they oxidize acetate.

Lipide and steroid metabolism.—Emmelot & Bosch have demonstrated in tumor slices and *in vivo* that acetate-C^{14} is converted into fatty acids and cholesterol and that glucose stimulates both processes, which indicates that the energy for these syntheses is furnished primarily by glycolysis (237). A lack of goitrogenic response to thiouracil in tumor-bearing rats is attributed by Begg to hypertrophied adrenals in the tumor-bearing rats; the hyperlipemia in tumor-bearing rats may be attributable to a pituitary factor (238). Wotiz *et al.* (239) have demonstrated the conversion of acetate into testosterone, androstenedione, progesterone, estradiol, and estrone in slices of a human testicular tumor.

Amino acids and proteins.—A kinetic analysis of the concentrative uptake of labeled glycine into ascites cells has been carried out by Heinz (240) who concludes that the amino acid must enter the cell in combined form and not by free diffusion. Von Euler *et al.* (241) have determined the levels of a number of amino acids in the Yoshida sarcoma, and Kit & Awapara call attention to the relative constancy of the free amino acid levels of various tumors (242). It has been found by Bassi (243), that whereas the amino acid compositions of liver and hepatoma proteins were the same, the latter had a greater resistance to heat flocculation. Kit has observed that the conversion of acetate into glycine by a mechanism, as yet undetermined, was eightfold higher in suspensions of the Gardner ascites tumor than in any normal tissues investigated (244). With the exception of hepatomas, Levintow (245) found the enzyme which converts glutamine to *p*-glutamoylhydroxamic acid to be low in concentration in tumors as compared with normal tissues.

The incorporation of several labeled amino acids into proteins of ascites cells was obtained under anaerobic conditions by Rabinovitz *et al.* (246), who concluded, on the basis of a lack of inhibition of this process by dinitro-

phenol, that glycolysis furnishes the energy for protein synthesis and that the amino acids are incorporated independently of each other. Weisberger *et al.* have found a greater incorporation of labeled cystine into human leukemic than into normal leukocytes both *in vivo* and *in vitro* (247). Busch & Greene have demonstrated the utilization of labeled plasma proteins to a much greater extent by tumors than by normal tissues (248) and they suggest that the utilization of proteins may be an important characteristic of growing tumors. Early in the experiment the highest uptake was in the tumor microsome fraction. Similar results have been reported by Gavrilova (249). Jablonski & Olson have measured the rapid biosynthesis of tumor lipoproteins (250).

Nucleotides and nucleic acids.—Schmitz and his colleagues (251, 252) have carried out extended anion exchange chromatographic analyses of the nucleotides in the acid-soluble fractions of Sarcoma 37 in ascites form and of the Flexner-Jobling carcinoma; they report few major differences from normal liver. Very sensitive analyses of the acid-soluble fractions of a number of tissues, including tumors, have resulted in Schneider's demonstration for the first time of the presence of free deoxyribosides in animals (253). De Lamirande *et al.* (254) have compared the composition of RNA nucleotides of livers and hepatomas and found an increased level of guanylic acid in the tumor. Analyses by Weymouth *et al.* (255) of thymus glands in normal, irradiated, and thigh-shielded irradiated mice (which do not develop lymphoid tumors) showed a marked elevation of RNA in the irradiated, tumor-susceptible group. A reduced DNA content of tumors in vitamin C-deficient animals was noted by Sokoloff *et al.* (256). Leibman & Heidelberger (257) have shown in liver and tumor slices and ascites cell suspensions that P^{32}-labeled nucleotides are dephosphorylated at the cell membrane, enter the cells as the nucleosides, and are rephosphorylated; therefore cells are not permeable to nucleotides.

A complete retention of the radioactivity in DNA in growing and partially hepatectomized rats has been demonstrated by Takagi & Potter (258) and by Kihara & Sibatani (259), which shows that during cell division the DNA is not broken down and reutilized. Backmann & Harbers have found different specific activities in two fractions of DNA (which differed in saline solubility) from the Walker tumor following P^{32} administration (260). The rates of incorporation of P^{32} into the nucleic acids of tumor-bearing rats have been studied by Khouvine & Montreuil (261). Harrington & Lavik have measured the incorporation *in vitro* of P^{32} into the nucleic acids of ascites cells and showed the process to be relatively resistant to irradiation (262).

Leibman & Heidelberger (263) have found that the utilization of uracil for nucleic acid pyrimidine synthesis is considerably greater in the Flexner-Jobling tumor than in liver (cf. 205); however, the utilization of uracil by intestinal mucosa was even higher. Lagerkvist *et al.* (264) have shown that incubation of ascites cells which labeled ammonia, aspartate, and ureidosuccinic acids results in RNA pyrimidine synthesis with dihydroorotic and

orotic acids as intermediates. Similar results were obtained *in vivo* by Anderson and her colleagues (265).

In a search for exploitable biochemical differences between tumors and normal tissues, Skipper's group (266) found that tumors utilized guanine to a much smaller extent than did normal tissues. The incorporation *in vivo* of glycine-2-C^{14} into the acid-soluble purine nucleotides of liver and Flexner-Jobling tumor has been studied by Edmonds & LePage (267), who found at short times after glycine administration that inosinic acid had the highest specific activity; they have discussed the interrelationship of the various nucleotides. Barclay & Garfinkel (268) have found that purine synthesis from glycine, *de novo*, predominates over the utilization *in vivo* of preformed adenine in sarcoma 180. A ribonuclease of different substrate specificity from the usual pancreatic enzyme has been found by Steckerl in ascites tumors (269).

Tissue culture.—The important strides made during the past year which have resulted in the determination of the nutritional requirements of mammalian cells in tissue culture, makes this an important technique for biochemical studies and merits inclusion in this review. Eagle (270, 271) has reviewed his work in this field with HeLa and mouse fibroblast cells and has listed the optimal concentrations of 13 amino acids, 8 vitamins, 6 salts, glucose, two antibiotics, and dialyzed serum proteins required for good growth. With this knowledge it is possible to apply the technique to studies of the role of the protein added to the nutrition of the cells, of isolations of new lines of cells for tissue culture, of antimetabolite mechanisms, of nutritional requirements for virus synthesis, of protein and nucleic acid biosynthesis, and of the biochemical interactions of normal and tumor cells grown simultaneously in the same culture. Caillaeu & Kirk (272) have studied the effects of horse serum and various media, more complicated than Eagle's, on the culture of L cells. Earle and his group have analyzed the free and conjugated amino acids in the protein-free ultrafiltrate of embryo extract that replaces the growth requirement for total embryo extract (273) and have demonstrated that ultrafiltrates from eggs will also replace the embryo extract requirement (274). Schleich has demonstrated that Yoshida sarcoma cells will grow in "symbiosis" with fibroblast cells in culture (275), and Leighton has found that tumors grown in culture on a cellulose sponge matrix tend to form patterns that resemble their normal histological appearance (276).

Although it is generally conceded that cancer is a metabolic disease, and it is consequently axiomatic that there must be metabolic differences between tumors and normal tissues, no such qualitative differences have yet been discovered in spite of intensive work. Nevertheless, it is imperative that the search be pursued with vigor.

Host-Tumor Relationships

Liver catalase.—Greenstein (277) has reviewed briefly the history of the well recognized fact that the liver catalase activity is lower in tumor-bearing

than in normal animals; this subject has received continued investigation. Lucké & Berwick (278) have found a close relation between the growth of ascites tumors and the reduction of liver catalase activity. A marked quantitative similarity between the reduction in content of liver catalase and formic acid oxidase in tumor-bearing mice has been discovered by Stein & Mehl (279). Nakahara & Fukuoka have studied the isolation of the substance, which they call "toxohormone," responsible for this effect and have produced an active dialyzable fraction by pepsin digestion of a nondialysable tumor extract (280); they have also reported the biosynthesis of "toxohormone" in tumor slices from arginine, phenylalanine, leucine, and ATP (281). Seabra & Deutsch (282) have shown that boiled extracts (*kochsafts*) of tumors decrease the activity of crystalline catalase *in vitro*, an effect also produced by aged liver homogenates. Cystine produces the same effect *in vitro*. In order to determine whether catalase activity, or concentration, or both is decreased, they have developed a new immunological assay for catalase. Endo *et al.* (283) have found that Deutsch's *kochsaft* factor which produces its effect *in vitro* is common to many other tissues, whereas "toxohormone" acting *in vivo* can only be obtained from tumors. However, two observations tend to throw considerable doubt upon the tumor specificity of this phenomenon. Heim *et al.* (284) have obtained liver catalase depression similar to that produced by tumors by injection of 3-amino-1,2,4-triazole. Day *et al.* (285) have obtained a similar effect by the injection of homogenates of normal mouse spleen and conclude that catalase depression is not attributable to a specific tumor hormone, but rather to a generalized stress reaction whose mechanism is not yet understood.

General metabolic effects.—Differences in the amounts of free amino acids in the livers of normal and tumor-bearing rats could not be found by Sassenrath & Greenberg (286), nor were significant differences observed in the tolerance to massive injections of amino acids; however, threonine dehydrase appeared to be lower in the livers of tumor-bearing animals (287). Babson & Winnick (288) have obtained evidence to suggest that protein is transferred from body tissues to the Walker tumor without going through the free amino acid stage (cf. 248, 249) and that nitrogen metabolism is not strictly a "one-way passage." Greenlees & LePage (289), in studying the metabolism of labeled ascites cells, reached a similar conclusion and showed that the protein metabolism approached the "one-way passage" state as the degree of anaplasia increased in a mammary adenocarcinoma which was undergoing progression from the benign to the autonomic state. Rodriguez *et al.* (290) have confirmed earlier reports on an increase in DNA in the livers, kidneys, and intestines of tumor-bearing rats without a concomitant increase of RNA. Bloor & Haven (291) have demonstrated that in tumor-bearing rats the weight of intestine relative to body weight was greatly decreased, which suggests that the amount of intestinal tissue is insufficient to support life and growth in the face of competition of the tumor. They have also found that feeding of dried Walker tumor to cachexic rats bearing

the same tumor, maintained appetite and lengthened the life span; the effect was attributed to the mixed phospholipides (292). Boyd *et al.* (293) have found a hydrolipotrophic effect of tumors upon the thymus gland. The reduction in liver uptake of fluorene-2,7-di-(sulfonamido-2′-naphthalene)-S^{35}, effected by tumors, is the subject of a study by Argus & Hewson (294). Cohen & Levi-Montalcini have found that the factor from sarcomas 37 and 180 which stimulates growth of nerve can be identified with the nucleoproteins of the microsome fraction (295); the factor is streptomycin-precipitable and stable to several proteolytic enzymes, but inactivated by digestion with trypsin and ribonuclease (296).

From the foregoing, it is clear that tumors exert a variety of effects upon the tissues of the host.

Multiple myeloma.—A considerable amount of new information has been made available about the large amounts of urinary and serum proteins produced by some patients suffering with multiple myeloma. Putnam and his group have shown that the Bence-Jones urinary proteins from different individuals have varied physical properties and N-terminal amino acids (297); by administration of N^{15}- and C^{14}-labeled glycine they have shown that the Bence-Jones proteins are synthesized much more rapidly than the elevated serum globulins and hence cannot be derived from them (298). In a characterization of the elevated serum proteins from a number of individuals by physical and N-terminal amino acid analysis Putnam concludes that these proteins are unnatural, and arise from a "perverted method of protein synthesis," resulting in a parasitism of normal metabolites diverted from normal pathways (299). Deutsch *et al.* (300) have determined the physical properties of two Bence-Jones proteins and have studied their immunological cross-reactions with normal γ-globulins. They conclude that these proteins and the "abnormal" globulins are related to the normal proteins and that the Bence-Jones proteins are fragments of the globulin molecules that may reflect a failure of methionine synthesis. Similar physical and immunochemical results have led Smith *et al.* (301) to the conclusion that these globulins are probably normal proteins that are present in excessive amounts and that they are not "abnormal." Slater *et al.* (302) have found that the serum proteins from a number of cases are all physically and immunologically specific, but related to normal γ-globulins. Differences in individual Bence-Jones proteins have also been revealed by salting-out analysis (303). Osserman & Lawlor (304) have reported that the elevated serum globulins contain bound carbohydrate, but that the urinary proteins do not; however, their data are not entirely consistent with this view.

Immunology.—The present unsatisfactory status of serological and other diagnostic tests for cancer is reviewed by Sprunt *et al.* (305). Korngold & Lipari have shown by diffusion analysis that a human tumor maintained for many generations in cortisone-treated rats, hamsters, and in chick embryo retains its human antigenic characteristics (306). Weiler has obtained antibodies, highly tissue-specific, from rabbits injected with rat

liver mitochondria and microsomes; these do not react with the corresponding particulates from azo dye-induced hepatomas; by use of fluorescent-tagged antibodies he has shown histologically the loss of the characteristic liver antigens during carcinogenesis before the appearance of tumor cells (307), thus demonstrating a protein deletion. The antibodies produced from hepatoma particulates reacted with several other tissue antigens; hence new antigenic characteristics appear to have been acquired. These results have important and obvious implications with respect to carcinogenesis and tumor characteristics. Shrek & Preston (308) have shown histologically that the sera of rats with spontaneous regression of Bagg's lymphosarcoma are toxic to the cells of that tumor *in vitro;* this effect is highly specific and implies an immunological response.

Rapport & Graf have obtained complement-fixing lipide haptens from the mitochondrial fraction of the Murphy-Sturn rat lymphosarcoma and have studied in detail their immunochemical properties (309); they are species- but not tissue-specific, since several tumors and normal tissues react with them. Darcy has analyzed by the diffusion technique the antibodies produced in rabbits from the sera of normal and tumor-bearing rats and found different antigenic structures in the two cases, the greatest effect being found in animals which the largest tumors; this antigenic difference might be developed into a diagnostic test (310).

A number of attempts to produce specific anti-tumor antibodies have met with varying degrees of success. Mason *et al.* (311) have injected melanin granules into rabbits and have obtained sera which agglutinate suspensions of these granules, a tissue-specific effect. However, studies with I^{131}-labeled antibodies *in vivo* resulted in no selective localization in the tumor. A partially purified I^{131}-labeled antibody to the Walker tumor showed a reaction *in vitro* with tumor as well as with other tissues (312). When injected into the animals, radioactivity was bound to a very small extent in the tumor but primarily was found in the adrenals and ovaries. The conclusion was reached that the antibodies were general anti-rat, rather than anti-tumor substances. Somewhat better results were obtained by Flax and his colleagues in similar experiments with the Ehrlich ascites carcinoma (313): the fractionated γ-globulin from rabbit anti-Ehrlich sera doubled the survival time of ascites-bearing mice (314). Using the same tumor, Colter *et al.* (315) found that rabbit antisera to the tumor DNA and RNA nucleoproteins prolonged the life of mice when injected at the same time as the tumor inoculum. Pollard & Bussell have found a chemically and genetically specific factor in the spleens of mice injected with carcinogenic hydrocarbons that inhibited the growth in tissue-culture explants of tumors induced by the same hydrocarbon; this is believed to be an antibody response (316). On the basis of these investigations a glimmer of hope for the immunological control of tumors may be discerned. In some interesting studies in human patients, the Grahams (317) have prepared antigens from tumors removed at operation, which in a few cases elicited complement fixation in the sera

of the same individuals. Similar extracts from noncancer tissues produced no such effect. If more reproducible antigens can be obtained and the results can be confirmed it would appear that humans could produce antibodies to their own tumors, a phenomenon that might eventually see clinical exploitation.

Green (318) has recently put forth an interesting and provocative hypothesis stating that carcinogenesis is an immunological phenomenon in which the chemical agent initiates a conversion of postulated tissue-specific "identity antigens" into tumor iso-antigens. Unfortunately space limitations preclude a discussion of the experiments quoted in support of the immunological character of carcinogenesis. Suffice it to say that none of them provide any evidence to this effect, and all of them can be explained more plausibly on other bases.

Chemotherapy

Screening.—In order to bring additional facilities to bear on the problem of cancer chemotherapy, the United States Public Health Service, the American Cancer Society, the Damon Runyon Fund, the Federal Drug Administration, and the United States Veterans Administration have united to sponsor a National Cancer Chemotherapy Service Center. An account of its inception has been written by K. M. Endicott, its chief (319). Stock has reviewed the techniques employed in screening compounds against animal tumors (320) and has edited a compendium of compounds that have given negative results in screening programs (321). Because of the wide variation in response of various animal tumors to the same drug and the general lack of agreement with the results obtained clinically, the problem of devising and selecting the best screening methods must be subjected to constant and critical scrutiny. One such effort involved a co-operative program sponsored by the American Cancer Society in which a number of laboratories tested identical batches of 27 compounds against a variety of biological and biochemical test systems in an effort to develop new screening methodologies. The program was coordinated by Gellhorn & Hirschberg (322), and the individual and collective results were published in a supplement to Cancer Research. The test systems used were: Sarcoma 180 (323), a spectrum of rat and mouse tumors (324), mammary adenocarcinoma RC (325), transplants of spontaneous mammary adenocarcinomas (326), mammary adenocarcinoma 755, glioma 26, and Brown-Pearce carcinoma (327), L1210 leukemias (328), Ehrlich ascites tumors (329), mouse viruses (330), bacterial viruses (331), bacteria and fungi (332), *L. caseii* (333), bacterial mutagenicity (334), aggregation of *Dictostelium discoideum* (335), developing frog embryo (336), developing chick embryo (337), growth and morphogenesis of *Drosophila* (338), P^{32} incorporation into nucleic acids *in vivo* (339), incorporation of glycine-2-C^{14} into Ehrlich ascites tumor cell purines and proteins *in vitro* (340), and the incorporation of P^{32} and formate into the nucleic acids of slices of Flexner-Jobling carcinoma and rat spleen (341).

While a great many results of interest to the individual systems were obtained, statistical analysis of the over-all data led to the conclusion that

> there is no single tumor which could be expected to select all useful agents and therefore a spectrum of tumors provides a greatly improved screening system. There is no evidence of any non-tumor system which could replace a tumor system as a screening tool for carcinostatic agents. This conclusion applies to the microbiological, developmental, and biochemical synthetic systems studied here (322).

This can be construed as further evidence for the unique metabolic properties of tumors.

Tarnowski & Stock (342) have discussed the factors leading to the choice of mammary adenocarcinoma RC as a supplement to sarcoma 180 in the Sloan-Kettering Institute's extensive screening program, but Scholler *et al.* found that primary spontaneous mammary adenocarcinomas are much less responsive to active tumor-inhibitory compounds than their first and second generation F_1 hybrid transplants (343). Dagg *et al.* have studied the effect of nitrogen mustard upon serial passages of human tumors grown in chick embryo (344). A technique which promises to be of interest for screening was developed by Baserga & Baum (345), who obtained blood-borne lung metastases following implantation of tumors in the tail of mice and subsequent amputation. Screening of several drugs in a single human subject by local injections into multiple cutaneous tumor nodules has been accomplished by Sullivan & Allen (346), a technique which may see wider application in the future. Those compounds reported to be active against experimental tumors during the past year have been listed in Table II.

Of considerable interest is the report of Oberhauser *et al.* (360) that in animals given large doses of potassium iodide and iodate there was a remarkably great inhibition of the growth of transplanted spindle cell sarcomas; independent confirmation of this finding is urgently needed. Goranson & Tilser (380) have shown that alloxanization of rats inhibited the growth and incidence of intraperitoneally, but not subcutaneously, implanted tumors and that, conversely, such intraperitoneally implanted tumors protected the animal against the diabetogenic effects of alloxan. According to Barach & Bickerman (381) a moderate degree of anoxia inhibits the growth of transplanted tumors. Helff (382) has obtained complete regression of spontaneous mammary tumors by extracts of histolyzed amphibian skin and gills, and Pikovski & Schlesinger (383) have found that heterologous transplantation of various strain-specific tumors was enhanced by prior injection of the lyophilyzed tumors. Zbarskii & Perevoshchikova (384) have demonstrated that histone administration inhibited the growth of a transplanted sarcoma. An increased survival time in animals bearing ascites tumors (385) and a growth inhibition of solid tumors in animals treated with ribonuclease has been observed by Ledoux (386).

The isolation from liver, lung, kidney, stomach, and tumors of tumor-necrotizing polysaccharides has been accomplished by Perrault & Shear (387), and the biological properties of the highly toxic, but effective tumor-

TABLE II

New Compounds Active in Screening Programs*

Compound	Tumor	Effect	Ref.
1-Isopropyl-5-methylazulene	E.A.	+	347
Purine and its riboside	T.C.	++	348
Pyridine-2-carboxaldehyde thiosemicarbazone and derivatives	L-1210	+	349
Amicetin	L-1210	+	350
Purine	S-180	++	351
Fluorocitrate	AC-755	+	352
Actinomycin C	S-180 RC	+ +	353
Diethylethoxy methylenemalonate	755	++	354
N-Methylformic hydrazide and derivatives	755	+	355
Acetic acid hydrazide	755	+	356
Derivatives of N-methylformamide	E.A.	++	357
Benzylidine derivatives of Puromycin	755	++	358
Ethylenediaminetetraacetic acid	W 256	+	359
Iodide and Iodate	Sarc.	++	360
Acenaphthenequinonebisulfite	S.Carc.	+	361
Sodium diethyldithiocarbamate	T.C.	+	362
2-Deoxy-D-glucose	K.A.	+	363
N-bis(β-chloroethyl)-alanine and derivatives	Y.	++	364
N-bis(β-chloroethyl) alanine oxide	Y.	++	364
3,4-Dimethyl-6-aminophenol and derivatives	Spon. Mamm.	++	365
Dinitrophenol	Sarc. Jensen	+ +	366 367
4-(p-Dimethylaminostyryl)-quinoline-methiodide	Lymph.	++	368 369
2-Methylthio-1,4-naphthoquinone and derivatives	T.C. E.A.	+	370
2-Methyl-4-carboxy-6-aminoquinoline	Y.	+	371
4-Aminopyrazolo(3,4d) pyrimidine and 1-methyl derivative	AC-755 L-1210	++ +	372
Furfurylacetone and derivatives	E.A.	++	373
γ-(p-Methoxyphenyl)-β-dichlorocrotonolactone	A.C.	+	374
Janus Green B	BS	+	375
2,5-Bis(ethyleneimino)-hydroquinone internal salt	Many	++	376
Chlortetracycline	Ovarian	+	377
Quinacrine	4 T's	+	378
N,N'-(4-methyl-m-phenylene)-bis-(aziridine-carboxyamide	W 256	++	379

* Abbreviations: E.A. for Ehrlich ascites; T.C. for tissue culture; W 256 for Walker 256 carcinosarcoma; K.A. for Krebs ascites; Y. for Yoshida ascites

necrotizing polysaccharide-lipide complexes from bacterial sources have been investigated by Creech & Hankwitz, who were unable to separate the toxicity from the necrotizing activity (388). Stern & Misirlija (389) have found that butazolidine induces temporary remissions in chicken leukosis, and Tanaka *et al.* (390) have noted that certain ascites cells in a tumor population are totally unaffected by podophyllotoxin and related compounds. Sparks *et al.* (391) have studied the activity of a number of compounds on a granulocytic chloroleukemia in rats.

A number of studies on the enhanced effect of combinations of chemotherapeutic agents have been reported. The antitumor effectiveness and toxicity of several combinations of cytotoxic alkylating agents have been studied in sarcoma 180 by Barvick & Goodson (392), and Garattini *et al.* (393) have shown that some acridines potentiate the activity of TEM against the Walker 256 carcinoma. Increased effectiveness of 8-azaguanine in combination with flavotin has been obtained by Woodside & Kelton (394), and Whitehead & Lanier (395) have potentiated the effect of local x-radiation of a lymphosarcoma by 1,3-dichloro-2-isopropyl-N-diethylcarbamate. Shapiro and his colleagues have continued their studies of combination chemotherapy, based on the idea of inhibiting enzymes that are present in low concentrations in tumors, by showing that testosterone augments the antagonism by deoxypyridoxine of various vitamin B_6 enzyme systems in tumors (396), and by the demonstration of synergism of ethionine and 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methyl pyrimidine against adenocarcinoma 755 (397). Peters *et al.* (398) have shown an augmentation of radium treatment of ascites tumors with Megaphen [N-(3′-dimethylamino)-propyl-3-chlorophenothiazine]. Hirono has determined that Yoshida ascites cells that had become resistant to nitrogen mustard N-oxide (Nitromin) were also resistant to nitrogen mustard and TEM (399). Abnormal mitoses were produced in the Walker tumor by urethan and were reversed by thymine, which led Boyland & Koller (400) to postulate that the drug may interfere with thymine synthesis by inhibiting transmethylation reactions; Koller has correlated the mitotic damage produced by 4-aminostilbenes with their growth inhibitory properties (401). Sugiura & Stock have studied the effects of various phosphoramides (402) and azaserine (403) against a spectrum of rat and mouse tumors, and Shay *et al.* (404) have investigated the effect of triethylenethiophosphoramide on a chronic myelocytic chloroleukemia in the rat. Jackson has shown that an irreversible resistance to TEM develops in the Walker 256 tumor (405), and a potentiation by Megaphen of the mitotic inhibitions caused by colchicine was found by Peters (406). A protection of tumors against local x-radiation by 2-mercaptoethylamine, which in itself had no effect upon the tumor, was reported by Neukomm *et al.* (407).

Druckrey (408) has studied the effect of TEM, nitrogen mustard, and Nitromin on the Yoshida sarcoma and was able to obtain complete regressions with the latter compound. He has analyzed his data in terms of dose-response curves.

The effects of N-methylformamide on mouse leukemias, solid tumors, and *Escherichia coli* have been investigated by Skipper *et al.* (409). They found marked tumor inhibition and observed that the *E. coli* inhibition was prevented by 2,6-diaminopurine and its riboside. Lettré *et al.* (410) have found, using P^{32}-labeled ascites cells, that the growth rate can be measured by determining the radioactivity; the effect of several drugs on this process has been measured.

Pharmacology and metabolism.—The work of Goldin and his associates provides an excellent example of the amount of quantitative biochemical information that can be gained from simple lethal toxicity experiments in mice when they are coupled with a biochemical awareness and a sophisticated use of statistics. They have analyzed the antileukemic specificity of aminopterin with reference to the optimal dosage regime for producing the greatest antitumor toxicity and the least host toxicity (411) and have shown further that the effectiveness of aminopterin can be increased by multiple administration, followed by citrovorum factor treatment (412). Similar criteria have been used to demonstrate that aminopterin is more effective against L1210 leukemia than amethopterin (413). The host-tumor-drug relationship and the degree of protection against the lethal toxicity of 6-mercaptopurine by various nucleotides and nucleosides have been determined (414, 415), and in contradiction to previous reports no synergism between amethopterin and 6-mercaptopurine against L1210 leukemia was observed, and, indeed, a detrimental effect in terms of toxicity to the host occurred (416).

In order to realize the full potentialities of a drug it is necessary to obtain some information about its distribution, excretion, and metabolism. Whereas research of this nature is ordinarily taken for granted in chemotherapy, until very recently there have been few examples in the realm of cancer chemotherapy. One such study, by Hamilton & Elion (417) on the metabolism of 6-mercaptopurine-S^{35} in man, has provided information on the blood-level and excretion of this drug and has shown that the majority of the urinary radioactivity could be accounted for as inorganic sulfate. The metabolism of C^{14}-labeled oxapentamethylenediethylenethiophosphoramide (OPSPA), a potent tumor inhibitory compound, has been compared in tumor-bearing rats and cancer patients by Heidelberger & Maller (418) who noted a considerable similarity between the blood-level and excretory patterns in the two species; considerable information was also presented on tissue distribution and biochemistry in the rats. The metabolism of triethylenephosphoramide-P^{32} in rats has been investigated by Craig & Jackson (419), who concluded on the basis of insufficient evidence that the drug itself was the primary excretory product. TEM, labeled in the ring with C^{14}, was shown by Nadkarni *et al.* (420) to be excreted in the urine primarily as cyanuric acid. In all the cases mentioned there was rapid urinary excretion and no selective localization of radioactivity in the tumor tissue, so that information on modes of action of these compounds will have to come from detailed biochemical studies of the nature of their metabolism and interaction with individual tissue constituents. Model experiments aimed in this direction have

been carried out by Wheeler *et al.* (421), who have studied the chemical interactions of nitrogen and sulfur mustards with purines, pyrimidines, and nucleotides. Trams & Klopp (422) have shown that transmethylations do not represent the major pathways of the metabolism of methyl-C^{14}-bis (2-chloroethyl)amine (HN2).

Biochemical effects of chemotherapeutic agents.—Potter's concept (423) of antitumor potentiation by two compounds that establish sequential blocks along a single metabolic pathway has been verified experimentally by Skipper *et al.* (424). They have found that ethionine, which by itself has no carcinostatic effect against L1210 leukemia, potentiated the activity of amethopterin, presumably by sequentially blocking the pathway of one-carbon metabolism, and that this potentiation could be reversed by administration of methionine. A similar augmentation of the effect of 8-azaguanine against mouse leukemia by 4-amino-5-imidazolecarboxamide, an inactive compound, was obtained by Mandel & Law (425).

A number of studies, including several already mentioned (339, 340, 341), have determined the effects of various chemotherapeutic agents on the incorporation of labeled precursors into nucleic acids. Way *et al.* (426) found that x-rays inhibited the incorporation of adenine into DNA, but that 8-azaguanine did not affect its incorporation into DNA or RNA. Barclay & Garfinkel (427) reported that N-methylformamide, though it stimulated the incorporation of formate into liver nucleic acid, inhibited incorporation into tumor DNA. Davidson & Freeman (428) observed that the alkylating agents: 1,4-bis(methylsulfonyloxy)butane (Myleran), nitrogen mustard, TEM, and triethylenephosphoramide did not affect the incorporation of P^{32} into DNA of adenocarcinoma 755, whereas 8-azaguanine and 6-mercaptopurine inhibited the process. A 90 per cent inhibition of the incorporation *in vitro* of glycine-2-C^{14} into the nucleic acid purines of the Ehrlich ascites tumor, with very low concentrations of azaserine, has been obtained by LePage *et al.* (429); Buchanan has demonstrated that azaserine blocks purine synthesis by preventing subsequent reactions of formyl glycine amide ribotide (430). Balis & Dancis (431) have found that amethopterin inhibits the incorporation of formate into nucleic acid purines and thymine in *breis* of leukemic spleen, but that *in vivo* only thymine was inhibited; similar results with purines *in vitro* were obtained with mouse leukemias (432) and human granulocytic leukemic cells (433) by Williams & Winzler. It is evident that in all these researches there is as yet no direct correlation between chemotherapeutic activity and the effect of compounds on nucleic acid biosynthesis. It will apparently not be possible to describe the complicated process of growth inhibition in simple biochemical terms until more knowledge is gained about the details of the mechanisms involved.

Nichol has found that the inhibition by amethopterin of the conversion of folic acid to citrovorum factor is less in resistant than in sensitive leukemic cells, but the reason for this has not been determined (434). A number of changes in the intracellular composition of leukemic cells in response to

antileukemic agents have been reported by Heaney & Eliel (435), but no least common denominator is evident.

In studies on the aerobic glycolysis of thymus lymphocytes, Miller (436) showed that nitrogen mustard stimulated the process, but triethylenephosphoramide did not affect it. Keyser (437) determined total blood serum polysaccharides and found a drop in their concentration in cancer patients with radiation- or nitrogen-mustard-induced remissions, which became elevated on relapse.

When Lettré (438) incubated Ehrlich ascites cells with nitrogen mustard, transplantability was lost, and when mitochondria from these cells were injected into animals containing the same tumor, growth was inhibited. This was interpreted as being the result of the entrance of "toxic" mitochondria into the tumor cells, but this view has not been widely accepted. Correlations between cytotoxic activity of several compounds and their cholinesterase activity were not found by Bullock (439). Waravdekar and co-workers have shown that the effect of α-peltatin on homogenates of sarcoma-37 resulted in a decreased glucose utilization and pyridine nucleotide content, with no change in the levels of aldolase, hexokinase, and adenosinetriphosphatase; acetylpodophyllotoxin pyridinium chloride in the same system decreased uricase and glutamic dehydrogenase without affecting adenosine deaminase and nucleoside phosphorylase (440). Ross & Warwick (441) have obtained a good correlation between the tumor inhibition of a series of nitrogen mustard analogues of the aminoazo dyes and the rate of reduction of the azo linkage by xanthine oxidase. Gross (442) has found that urethan inhibits tissue adenosinetriphosphatase. An alteration in the pattern of free amino acids in liver by the presence in the animal of a tumor has been reported by Levy *et al.* (443), who found a further change in animals which were force-fed ethionine. Brockman *et al.* (444) observed that whereas acid hydrazides produced little tumor inhibition in mice fed a normal diet, there was considerable inhibition in animals maintained on a vitamin B_6-deficient ration.

It is apparent from the foregoing that the various drugs exert their effects by different mechanisms, and it is only in the cases of the antifolics and antipurines that some understanding of their mechanisms of action is at hand.

Summary

During the past year there has been a strengthening of the concept of the essentiality of protein-binding in the mechanism of chemical carcinogenesis, and the elucidation of the structure of part of the dibenzanthracene-protein complex has been accomplished. It is now established that the pituitary gland is not essential for carcinogenesis, and progress has been made in our understanding of environmental cancer and radiation leukemogenesis. Remarkable new claims for the virus etiology of mouse leukemias have been made. The nutritional requirements of mammalian cells

in tissue culture have been worked out, and new information suggests that the proteins of multiple myeloma are related to normal globulins. Some progress has been made in immunizing animals against transplanted tumors, and the suggestion has been made that humans form antibodies to their own tumors. Increased activity in chemotherapy has centered around the evaluation of screening methods, but no new clinically effective drugs have been discovered.

In spite of intensive work, the essential biochemical lesions of cancer remain unknown. New ideas and approaches are urgently needed to solve this all-important problem.

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CHOLESTEROL METABOLISM[1,2]

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In this review we have attempted to scan the field of sterol metabolism from the vantage ground of the quantitatively most important animal sterol, cholesterol. If we have emphasized views in agreement with our own it has been intentional, so that the reader may not feel as though he were balancing opposing observations while the truth awaits determination by the addition of a single hair to either side.

We have been forced to omit treatment of many topics. Among these are methods of analysis and their influence on data, carcinogenic activity, atherogenesis, structure and distribution of sterols, and microbiological degradation and synthesis. The section on steroid hormones is severely curtailed.

The Biosynthesis of Cholesterol

Tissue sites.—With the possible exception of mature nerve tissue, every animal tissue adequately investigated seems capable of incorporating labelled acetate into cholesterol (114). Sites recently demonstrating this ability include skin of humans and monkeys (77, 213), aorta of various animals (89, 307), dog ovary (233), and erythrocytes of rabbit and man (7, 192, 259).

Intracellular site.—An enzyme system associated with cytoplasmic formed elements which sediment from liver homogenates at about 30,000 g is capable of synthesizing cholesterol (52, 53, 54, 85, 95, 170, 206). Lysis of such elements and subsequent centrifugation can result in particle-free extracts which will synthesize cholesterol (235, 254). Stable powders may be prepared from such extracts by lyophilization (84) or acetone-drying (229). Upon redissolving, these stable soluble enzyme systems are capable of cholesterol synthesis from added precursors (230, 231, 234, 253).

Reactions.—A reaction scheme has been assembled from the treatment of portions of this field by Bloch (34, 36), Schwenk *et al.* (259), Rudney & Farkas (255), Coon (70), Bonner (42), Rabinowitz *et al.* (232), Gould (123), Fukushima & Rosenfeld (116), and Cornforth (72). The reactions making up this scheme, although actually observed in various *in vitro* and *in vivo* experiments, are not proven to involve obligatory intermediates of cholesterol anabolism in the intact animal. Many intermediate reactions and energized forms of compounds have been omitted. Each compound should be considered as possibly being in equilibrum with other compounds of like number of carbon atoms, not necessarily indicated in the scheme. The scheme, grouped into four stages, is given below. No completely adequate proof of the

[1] The survey of the literature pertaining to this review was completed in November, 1955.

[2] The following abbreviations are used in this review: ACTH for adrenocorticotropin; CoA for coenzyme A; DOC for deoxycorticosterone.

necessity for any single reaction is available but a great deal of evidence is consistent with the scheme, and a resume is included here of some of the evidence cited above, with emphasis on more recent contributions.

Stage 1. Biosynthesis of Branched Chain Fatty Acids from Acetate:—Acetyl CoA (67, 174) condenses with itself to form acetoacetic CoA which, with an additional acetyl group, yields β-hydroxy-β-methyl glutarate. The glutarate decarboxylates to β-hydroxy-isovalerate which loses water to yield ββ'dimethyl acrylic acid. This five-carbon compound is considered to be an isoprenoid monomer (11, 34, 42).

Dependency of these reactions on CoA is suggested by the experiments of Klein & Lipmann (171, 172) and Boyd (44) who found parallels between sterol synthesis and CoA levels. Brady & Gurin (45), Curran (78), and Zabin & Bloch (315) implicate acetoacetate as an intermediate in sterol synthesis. Rabinowitz & Gurin show a purified system to require CoA for synthesis of β-hydroxy-β-methyl glutarate from acetate. Johnston *et al.* (155) have shown conversion of acetate to acetoacetate and β-hydroxy-β-methyl glutarate by a CoA dependent reaction. Coon *et al.* (71) have shown the steps in Stage 1 to occur as part of the animal degradation of valine. Rabinowitz & Gurin (235) found that acetyl CoA incorporation into cholesterol was reduced in the presence of β-hydroxy-β-methyl glutarate and ββ'dimethyl acrylic acid, and Rabinowitz (230, 231, 234) has reported synthesis of all the above compounds, including branched chain acids, from acetate. The same acids are synthesized from acetate by plants (21). Phillips & Langdon (222) report that monofluoroacetic acid is efficiently used as a substrate for the biosynthesis of fluorine-containing nonsaponifiable lipides.

Stage 2. Condensation of Isoprenoid Monomer to Squalene:—Two molecules of the isoprenoid monomer condense to a substance similar to, but not identical with, geranic acid (255). The likelihood that dimethyl acrylic acid is this monomer is suggested by its probable polymerization to long chains in some plants (11, 42, 43, 298), by its distribution of carbon derived from acetate (72), and by the finding that it is efficiently incorporated into cholesterol with a distribution of label in cholesterol in agreement with theory (38).

Rudney & Farkas (255) have attempted to find the 10-carbon intermediate postulated to result from the condensation of two molecules of ββ'-dimethyl acrylic acid and succeeded in trapping the compound with the use of geranic acid, but were not able to identify it as geranic acid. It thus appears likely that a compound with properties similar to geranic acid will prove to be the postulated intermediate. It appears likely, from the work of Dituri *et al.* (84), who found that farnesol reduced incorporation of acetate to squalene, that a 15-carbon intermediate is also involved. Later work from this group (232) showed that acetate radioactivity could be recovered in farnesinic acid but not in farnesol itself. The suitability of farnesinic acid for production of squalene by "head-to-head" condensation follows from the pattern of distribution of 2-C^{14} acetate into squalene determined by

Cornforth & Popják (75). An actual tracer experiment, however, has not been reported as yet.

State 3. Squalene en Route to Cholesterol:—Much tracer evidence has accumulated showing incorporation of squalene into cholesterol. Thus Langdon & Bloch (181) fed inactive squalene to rats together with radioactive acetate and recovered radioactive squalene from the liver. This labeled squalene was then fed to other rats, and C^{14}-cholesterol was recovered (182). Liver slices from these rats showed depressed incorporation of 1-C^{14} acetate into cholesterol, presumably because of preferential incorporation of the fed squalene. Similar results were obtained by Tomkins *et al.* (296). Langdon (180) felt that the conversion of squalene to cholesterol by mice was efficient (10 per cent of administered squalene carbon was recovered as cholesterol) and therefore could not involve breakdown of squalene to small units.

Eidinoff *et al.* (88) have found 0.1 per cent of radiocarbon administered to a dog as acetate-2-C^{14} to be present in the liver as squalene. This squalene was recovered as cholesterol when given to mice, with less than 1 per cent of the radio-carbon appearing as CO_2 and hence suggesting that very little squalene is broken down to smaller units. Perhaps this small amount of CO_2 may be formed from the three carbons lost in the ultimate conversion to cholesterol. Diturit *et al.* (84) have isolated C^{14}-squalene from a particle-free extract of rat liver incubated with either C^{14}-acetate or methyl-labeled β-methyl-β-hydroxy-glutaric acid. The latter was a more efficient precursor. The active squalene produced in this particle-free system was efficiently converted to cholesterol when transferred to a fresh system of the same type. McGuire & Lipsky (200) have observed that the feeding of squalene decreased the rate of incorporation of acetate-1-C^{14} into plasma cholesterol in patients. No depression occurred if the acetate was given before the squalene. Synthesis of cholesterol from acetate by human skin was found by Nicolaides *et al.* (213) to be accompanied by synthesis of squalene of specific activity 10 times higher than that in the sterols, emphasizing squalene as a probable precursor.

Despite the evidence cited above showing that squalene can arise from acetate and be converted to cholesterol, there is still some doubt as to its place in cholesterol biosynthesis. Thus, Popják (227), though showing that liver slices and hens' ovary membranes form both squalene and cholesterol from acetate, believes the rate of incorporation of squalene is too slow for this hydrocarbon to be a true intermediate and raises the possibility that the true intermediate may be some substance with which squalene is in equilibrium.

In order for squalene to give rise to cholesterol, it is necessary that the molecule be folded to the same arrangement as the sterol skeleton and cyclized to yield the required ring structure. Several theoretically equivalent schemes have been proposed by which this might come about (72). A choice between these schemes is made possible by degradation studies. By this means, the

derivation of the individual carbon atoms of cholesterol and of squalene from methyl or carboxyl carbon atoms of acetate has been proven (72). This fundamental work, to which Bloch and his co-workers (35, 39, 40, 310, 312) and Cornforth, Popják *et al.* (65, 72, 74, 150, 226, 227) have made impressive contribution, has established a frame of reference by which the fitness of postulated cholesterol intermediate reactions may be tested. The distribution of "methyl-derived" carbon atoms in squalene biosynthesized from 2-C^{14}-acetate has been determined by Cornforth and Popják (226). To decide between postulated cyclization schemes it is necessary to determine which hypothesis will best explain the known distribution of acetate carbon in cholesterol. When subjected to this test (72, 80, 310) the scheme of Woodward & Bloch (310) agrees with the actual pattern of distribution of acetate carbons in cholesterol, while other schemes do not.

Stage 4. Transformation of Sterol Precursor to Cholesterol.—If the squalene molecule is cyclized as suggested by Woodward & Bloch (310), the required sterol skeleton would emerge in the form of the triterpenoid alcohol 4,4′,14α trimethyl-cholesta-8,24 diene-3β-ol (lanastadienol or lanasterol) except for the presence of a methyl group attached to C-13, which could presumably be provided by rearrangment during the cyclization.

Additional evidence for the participation of the lanastadienol is furnished by the work of Clayton & Bloch (69) who have isolated this alcohol containing significant amounts of C^{14} from rat liver homogentates incubated with C^{14}-acetate. The yield of radioactive lanastadienol was much enhanced by incubation with an ultrasonic dispersion of mixed wool fat sterols, which presumably furnished an equilibrium mixture of intermediate sterols. When C^{14}-lanastadienol was incubated with the homogenate, C^{14}-cholesterol and other more polar sterols were recovered. Loss of three methyl groups from the lanastadienol would result in the formation of zymosterol. Meyer (204) has suggested that an oxidative demethylation mechanism may be active in the removal of the quaternary methyl groups of lanastadienol, since a similar mechanism allows human placenta to convert androgens to estrogens. Schwenk *et al.* (262) have fed radioactive zymosterol to rats and recovered radiocholesterol. The position of zymosterol as a possible intermediate is strengthened by their finding that the sterols produced by resting yeast from 1-C^{14}-acetate may contain zymosterol more active than the end-product, ergosterol.

Other sterols which may be close precursors to cholesterol include Δ^7-cholestenol and 7-dehydrocholesterol. Fieser (94) has found Δ^7-cholestenol as a frequent contaminant of "pure" cholesterol prepared from animal sources. Langdon & Bloch (183) and Tomkins *et al.* (296) have suppressed the incorporation of C^{14}-acetate into cholesterol in rats by administration of Δ^7-cholestenol or 7-dehydrocholesterol. Presumably this resulted from dilution of radioactive precursor by the inactive Δ^7-cholestenol. Kritchevsky (175) and Lemmon *et al.* (185) fed rabbits a diet containing 1 per cent of Δ^7-cholestenol and observed increases in serum lipoproteins and cholesterol

identical with those produced by cholesterol feeding alone. The unsaturated sterol did not accumulate as such in the rabbit serum. Wells & Baumann (305) also found injected Δ^7-cholestenol to disappear rapidly from the blood of rats. Biggs *et al.* (32) isolated tritium-labeled cholesterol from the liver sterols of rabbits fed tritium-labeled Δ^7-cholestenol. Tomkins *et al.* (297) found that the feeding of Δ^7-cholestenol, 7-dehydrocholesterol, or of cholesterol to rats resulted in diminished ability of the surviving liver slices to incorporate acetate 1-C^{14} into cholesterol. Since other sterols not thought to be metabolic intermediates, such as cholestenone and dehydroisoandrosterone (207, 305), also suppressed incorporation of acetate into cholesterol, the results serve also as reminders of the difficulty of proving a principal metabolic pathway even with the help of tracer materials.

The sterol precursors of cholesterol and their metabolic sequence remain uncertain, although their isolation and identification from the high counting companions (HCC) of cholesterol biosynthesis being investigated by Schwenk *et al.* (260 to 263) is probably to be expected within the next year.

Structure of Sterols

A stereo-chemical correlation of the absolute configuration of cholesterol with that of D-glyceraldehyde has been worked out by Cornforth *et al.* (76) and also by Riniker *et al.* (240). The conventional projection of the steroid molecule therefore accurately represents the absolute configuration.

Inhibition of Synthesis

Steinberg & Fredrickson (284) have reported α-phenyl-N-butyrate and analogous compounds to inhibit incorporation of acetate into cholesterol by rat liver slices.

Competitive Inhibition of Sterol Utilization

While plants and animals can synthesize sterols from small molecules, some insects at least cannot (215) and require a source of dietary sterol for normal growth and development. This has allowed Noland (215, 216) and also McKennis (201) to determine facts concerning competitive inhibition of cholesterol utilization by structural analogues which may well prove important in the study of mammalian sterol metabolism. The most potent inhibitors found were thiocholesteryl acetate, cholesteryl chloride, cholesteryl methoxide, and thiocholesterol.

Rate of Synthesis of Cholesterol

Critique of radioactive tracer methods.—The fundamental theoretical relations involved in the study of metabolic turnover rates by means of isotopic tracers have been treated by Reiner (237, 238) whose papers, at the time this is written, apparently remain the only adequate exposition. While isotopic tracers can be used with comparative ease to trace qualitative metabolic pathways and to demonstrate activity in quantitatively con-

stant body components, determination of the rate of this activity is far more difficult. Indeed, there is still disagreement as to the proper definition of "turnover" (169, 199, 316). Turnover concerned with a remote precursor directly connected with more than one product is especially difficult to ascertain. When the central position of acetyl-CoA in an immensely complicated network of fat, carbohydrate, and protein metabolism is recalled, it is easily seen that many of the studies which claim to determine rate of synthesis of cholesterol by labeled acetate uptake actually provide little more than information on the per cent of labeled acetate which remains incorporated in sterol at a specific time in a specific experiment.

The use of rate of precursor incorporation as an index of rate of synthesis requires a knowledge of the concentration of the labeled precursor substance over the time of the experiment. This knowledge is not always available when acetate is administered as a single injection (15, 16, 98). Indeed the studies of Schwenk *et al.* (263) and Dayton *et al.* (83) showing that incorporation of acetate into cholesterol is complete in the liver within less than 10 min. after administration, suggest that the concentration of precursor substance changes drastically and rapidly over the course of all experiments dependent on only one or a few injections of labeled precursor.

The availability of the acetate pool may be more constant when acetate is fed with the ration rather than administered as isolated injections. However, Pihl *et al.* (223) found the isotope concentration of acetyl groups to change with time even when this method was employed.

The use of deuterium or tritium allows tagging of all the body water at a constant level and hence provides for a physiologically inexhaustible supply of labeled small molecules. Isotopic hydrogen, however, is susceptible to exchange reactions to an unknown extent. This exchange is reflected in the generally slower turnover rates for cholesterol found with the use of isotopic hydrogen as compared with those found with isotopic carbon. Provision of a physiologically inexhaustible supply of precursor small molecules also may be achieved by the use of tissue sections, homogenates, or extracts since the amount of added labeled precursor probably saturates the reaction capacity of such preparations. However, rates and intermediates determined by experiments with isolated preparations may be subject to many complex rate disturbing influences.

Another difficulty in determining the rate of cholesterol synthesis arises from the probability that the intrinsic synthesis rate varies for different tissues and different types of cells with a tissue. For instance, Saba & Hechter (256) suggest that there are at least two different pools of cholesterol in the adrenal cortex.

Still another uncertainty occurs because of the rapid molecular exhange of cholesterol between cells and body fluids. This exchange depends, as Eckles *et al.* (87) have pointed out, not on the rate of synthesis of cholesterol but upon the availability of the specific proteins to which cholesterol is bound and their relative affinities for it.

When in addition to these difficulties, it is recalled that both the digitonin precipitation and the Liebermann-Burchard reaction generally used to quantitate cholesterol are nonspecific, it is not surprising to find that considerable divergency exists in the literature concerning cholesterol synthesis rates. For example, the half time of regeneration of plasma (=liver) cholesterol in the dog has been published by various observers (87, 129, 166) as being from 7 to 144 hr., in human subjects (177, 191, 200) from 14 to 312 hr., and for the rat (147, 151, 179, 223) from 24 to 144 hr. Similar divergencies have been noted in the reports of different observers studying animals in abnormal states. Such divergencies both in normal and abnormal animals, make one hesitant concerning tracer technique as an infallible index to rate of cholesterogenesis. The reports of rate of cholesterogenesis under various conditions listed below are given with the above qualifications in mind.

Rates of Cholesterogenesis in Various States

Effect of diet.—It appears that fasting markedly reduces the rate of cholesterol synthesis in the rat as measured by tracer technique (300). The feeding of either carbohydrate alone (143) or fat (5, 143) to the fasted rat tends to correct this deficiency. A high fat diet increases the rate of cholesterol synthesis (308). The effect of feeding of excess cholesterol upon cholesterogenesis is not certain. In the dog the observations of Gould *et al.* (125) suggest a depression of rate of synthesis subsequent to excess cholesterol feeding, but Mosbach & Abell's observations (207) are not in conformity with such a view. In the monkey Cox *et al.* (77) found cholesterogenesis depressed by cholesterol feeding. In the human subject it recently has been observed (295) that the feeding of excess cholesterol did not affect the rate of cholesterogenesis.

Deranged endocrine states.—The effect of the diabetic state upon cholesterogenesis is uncertain. Thus Hotta, Hill & Chaikoff (146) observed an actual increase in cholesterogenesis in the diabetic rat which could be alleviated by the feeding of fructose (148). On the other hand, Hutchens & Yamada (152) found a normal rate of cholesterol synthesis in the diabetic rat.

Rosenman *et al.* in a series of studies in the last several years (63, 245, 249) employing various techniques, demonstrated that the rate of hepatic synthesis of cholesterol in the rat varied directly with the activity of its thyroid gland (i.e., a high rate of synthesis in hyperthyroidism and the converse in hypothyroidism). Their findings have been confirmed in the rat by Marx *et al.* (197) and Dayton *et al.* (81) and in man by Kurland *et al.* (178), Lipsky *et al.* (190), and Gould *et al.* (124).

The rate of cholesterogenesis in the hypophysectomized rat appears to be unchanged if provision is made for adequate intake of carbohydrate (143).

Hypercholesteremic states.—Friedman & Byers (56, 59, 100) came to the

conclusion that the accumulation of cholic acid in plasma occurring after biliary obstruction initiated the hypercholesteremia. The mechanism by which plasma cholate effected this hypercholesteremia was not dependent upon a change in hepatic rate of synthesis or discharge, as measured by incorporation of tritrium into cholesterol, but upon apparent "trapping" of cholesterol in the plasma. An increase of plasma cholate also was found in human individuals suffering from various other types of hypercholesteremia (107).

Fredrickson *et al.* (98) employing 1-C^{14}-acetate believed that the rat subjected to biliary obstruction exhibited an increase in its hepatic rate of synthesis of cholesterol. This is contrary to the findings of Landon & Greenberg (179) who also found the half life of liver cholesterol in the rat to be much lower (22 hr.) than that reported by Fredrickson *et al.* This half life period could adequately explain the plasma cholesterol accumulation after biliary obstruction if there were an interference with the usual return of cholesterol into the liver as postulated above (56, 59, 100). There is then no necessity for postulating an increase in the liver synthesis and discharge of cholesterol into the plasma in this disorder.

Hypercholesteremia secondary to triton injection.—Kellner *et al.* (167) studying the possible hypercholesteremic effects of various surface active substances observed that injected Tween 80 and Triton A-20 were potent hypercholesteremic agents, but they did not determine their mode of action. Later Friedman & Byers (99), confirming the hypercholesteremic effect of triton, also found that this substance did not appear to alter the rate of hepatic discharge of cholesterol but, like cholate, effected "trapping" of cholesterol in the plasma and only in the plasma. Waddell *et al.* (302) also observed that triton markedly inhibited the usual rate of disappearance from plasma of injected lipide.

Frantz & Hinkelman (96), however, employing labeled acetate, concluded that a threefold increase in rate of synthesis of cholesterol had occurred and was possibly responsible for the hypercholesteremia observed. There are several serious objections to this concept. First, if the excess cholesterol in plasma were attributable only to an increased rate of discharge of cholesterol from the liver, then such excess plasma cholesterol should result in an increased deposition of hepatic cholesterol within a few hours, as is invariably found whenever excess soluble cholesterol is injected (104). However, this did not occur during the period of plasma cholesterol rise but only after the plasma cholesterol had begun to fall after 72 hr. But it is precisely between 72 and 96 hr. that a single injection of triton also disappears from the blood (73). In other words, as was found by Friedman & Byers (99), the hypercholesteremia attendant upon the injection of triton is conditioned by the concentration of triton in plasma and not upon its possible effects upon hepatic parenchyma. Secondly, as pointed out above, their calculation of the half life of liver cholesterol in the normal rat is in sharp disagreement with that observed by Landon & Greenberg (179). Thirdly, the inhibiting effect of heparin upon triton induced hyperchol-

esteremia (99) suggests that the hypercholesteremic effect of the latter substance is mediated within the plasma itself. Fourthly, even if there really were an increase in the rate of hepatic synthesis of cholesterol after triton injection, this increase could well be a secondary phenomenon arising because of a relative failure of the liver to receive its usual increment of plasma cholesterol, similar to that which may occur in the animal deprived of exogenously derived cholesterol. In view of these objections it seems to the reviewers that the prime mode of triton action must reside in the plasma itself. Here the plasma cholesterol increment is so altered, perhaps by alteration of lipoproteins, that cholesterol is partially "trapped" and accumulation proceeds.

Nephrotic hypercholesteremia.—Recently, the syndrome produced in the rat by injection of antikidney serum has been shown by Heymann & Lund (142) to mimic closely clinical nephrosis. This discovery has made the study of lipide metabolism in the nephrotic syndrome a somewhat easier task. Rosenman *et al.* (61) employing this method, studied the cholesterol and lipide metabolism of the nephrotic rat. They found, as noted by London *et al.* (193) in his study of the nephrotic patient, that no increase in hepatic rate of cholesterol synthesis occurred in the disorder. Furthermore, they observed that the hypercholesteremia was of endogenous origin (112), that it was independent both of the intestinal absorption and excretion of cholesterol (64, 250), and, finally, that the cholesterol derangement (i.e., its excess accumulation) was confined to the blood alone (251). These authors also observed (252), as did Kleinerman (173), that the injection of heparin markedly ameliorated the nephrotic process, being particularly effective in reducing or preventing both hypercholesteremia and hyperlipemia. On the basis of these experimental observations, Rosenman *et al.* concluded the nephrotic hypercholesteremia was primarily a result of the "trapping" of cholesterol in plasma. Recently they have shown (248) that the urinary loss of of albumin occurring in the nephrotic syndrome is the initiating cause of both the hypercholesteremia and the hyperlipemia, a finding long suspected clinically. This hyperlipemic effect of albumin loss may possibly be mediated by its function as acceptor of fatty acids released in the "clearing" process (213, 219, 220).

Absorption of Cholesterol

Sources of intestinal cholesterol.—The cholesterol which is absorbed from the intestine comes from that present in the food, in the bile, and in the juices excreted by the intestine itself. Of these three sources, bile itself contributes the least (105). Thus in the rat, seven times as much cholesterol is excreted by the intestinal wall itself (60, 110) as enters into the intestinal lumen via bile. Moreover, both the rabbit and rat excrete more sterol into their intestinal lumen than they receive from stock laboratory foods (101).

Site of Absorption of Cholesterol

Although various earlier investigators believed that cholesterol was absorbed from both the small and large bowel, their measurements were

indirect. Yamakawa *et al.* (313) were the first to analyze the intestinal lymph itself, and they observed no increase in cholesterol absorption when cholesterol was placed in the large bowel, suggesting that such absorption occurred somewhere in the small bowel. This has recently been confirmed (110).

Processes Involved in and Affecting Absorption of Cholesterol

It has long been known that bile salts, esterifying enzymes, and fats are intimately associated with the absorption of cholesterol. Besides the possible influence of these substances, the amount of cholesterol absorbed in any given circumstance is dependent upon the amount of cholesterol eaten and the amount of possible interfering substances also ingested.

Bile salts.—Although the absolute necessity of the presence of bile and bile salts for the intestinal absorption of cholesterol has long been known (208, 276) and again more recently confirmed (41, 60), little information is yet available as to their mode of action. Recently Swell *et al.* (291) referring back to the observations of Nedswedski (209) have postulated that bile salt aids in the esterification of cholesterol (by pancreatic esterase) by first combining with cholesterol. However, before it can be assumed that bile salt promotes the absorption of cholesterol solely by aiding in its esterification, it will be necessary to demonstrate that esterified cholesterol can be absorbed in the absence of bile. Until this critical experiment is done perhaps it would be best to continue to believe that bile salt promotes cholesterol absorption because of its surface active and emulsifying properties.

Esterifying enzyme.—Although the major fraction of cholesterol is esterified in its progress from the lumen of the intestine to the intestinal lymph vessels, neither the source of the enzyme nor the precise site of esterification have been determined.

Swell *et al.* (290) found that the intestinal mucosa of rats exhibited considerable esterifying activity in the presence of bile salt. The properties of this enzyme so closely resembled those of the esterifying enzyme in pancreatin that the authors postulate that it probably arose from the pancreas and was then absorbed and stored in the intestinal cells.

However, other investigators (57, 281, 299) with the exception of Hernandez *et al.* (140) have not observed profound changes in cholesterol absorption after either diversion of pancreatic juice from the intestine or removal of the pancreas. In the experiments of Hernandez *et al.* a preparation was employed in which the duodenum of the rat was flooded with enormous volumes of a dilute bile obtained from a donor rat subjected to extreme hydration. The quality of such a bile and its possible abnormal influence upon intestinal absorption make the conclusions derived from such observations of doubtful application in the present instance.

With regard to the site of esterification, Swell *et al,* (288a) observed that when fed cholesterol esters and bile salt, rats exhibited a lower blood chol-

esterol than when free cholesterol and bile salt was fed. They used this as evidence for the hypothesis that cholesterol must be absorbed in the free form and then esterified within the mucosal cells prior to its entrance into the intestinal lymph. Recently Hernandez *et al.* (139) also hypothesized that esterification occurred within the intestinal cell after they had observed that when both epicholesterol and cholesterol were fed to rats, cholesterol was absorbed twice as rapidly as epicholesterol, and about half of the former and none of the latter was esterified. These observations, however, suggest to the reviewers that cholesterol may be absorbed at least in part without the necessity of esterification. Recent experiments of Daskalakis & Chaikoff (79) also support this conclusion.

Fats.—The absorption of cholesterol is conditioned by the quality and quantity of fat present in the intestinal lumen. Swell *et al.* (292) found little or no differences in the blood levels of cholesterol in rats fed various neutral fats, but apparently the ingestion of free fatty acids (stearic, oleic, and linoleic) led to higher blood cholesterol levels. Later, (288) these authors observed that ingestion of a relatively unsaturated soybean oil led to a higher blood cholesterol than the ingestion of a more saturated one, a finding opposed to those of Kinsell and other workers detailed below. Swell and his associates have attempted to explain their observations by theories dealing with changes in intestinal cholesterol absorption mediated by fats and fatty acids. However cholesterol changes in blood cannot be used as an indicator of changes in cholesterol absorption. The blood cholesterol at any given instant is the resultant of many factors (62) the least of which may be the intestinal absorption of cholesterol.

A possible major advance in the study of cholesterol metabolism was made when Kinsell and his associates (115, 168) observed that the administration of unsaturated vegetable fats in large amounts (30 or more per cent of the total diet) led to a profound fall in the plasma cholesterol and phospholipide level of both normal- and hypercholesteremic individuals. These studies have been confirmed in man by Beveridge *et al.* (30) and Ahrens *et al.* (2). They strongly suggest that the "hypocholesteremic effect" of an ingested fat is directly proportional to its degree of unsaturation. The mechanism responsible for this hypocholesteremic effect is unknown, but it does not appear to be attributable to a decrease in cholesterol absorption (164, 176).

Dietary cholesterol.—In the rat Friedman *et al.* (34) found that the absolute intestinal absorption of cholesterol as measured by analysis of intestinal lymph increased when excess cholesterol was ingested, but the fraction absorbed of the total amount ingested was found to diminish with the dose administered, a finding confirmed by Lin *et al.* (188).

Possible interfering substances.—(*a*) Sitosterols: Sperry & Bergmann (277) observed in 1937 that sitosterol administration decreased the hepatic cholesterol content of mice, and Peterson (221) reported in 1951 that sitosterols protected the cholesterol-fed chick both from excessive hyperchol-

esteremia and liver deposit of cholesterol. This observation stimulated the many subsequent studies upon the possible effects of sitosterols upon cholesterol absorption, plasma cholesterol level, and degree of atherosclerosis in cholesterol-fed animals including man. As might be expected, there is not complete agreement either as to the effectiveness of sitosterol administration or as to its possible mode of action.

Although Hernandez *et al.* (141) found that the administration of mixed soy sterols led to a marked reduction in cholesterol absorption as measured by analysis of intestinal lymph after ingestion of cholesterol-4-C^{14}, a study by Rosenman *et al.* (246) failed to reveal a significant reduction in cholesterol absorption after ingestion of soy sterols. Hernandez *et al.* (138) restudying the question, again found impairment of cholesterol absorption when soy sterols were given, but apparently far less impairment was found in this second study. Moreover, although they criticized the methods of chemical analyses employed by Rosenman *et al.*, their use of the same methods gave results comparable with those that they obtained using radioactivity technics of measurement. Recently Friedman *et al.* (113) restudied the problem employing β-sitosterol. Little or no effect of sitosterol administration upon cholesterol absorption was observed. Beher & Anthony (17), however, find β-sitosterol feeding protects mice against dietary cholesterol accumulation in the liver.

Besides the uncertainty concerning the possible inhibiting effect of sitosterol upon cholesterol absorption, there is considerable disagreement as to its effect upon the blood cholesterol level. Thus, although Pollack (228) found that sitosterol administration markedly protected the cholesterol-fed rabbit against hypercholesteremia and atherosclerosis, Dreisbach *et al.* (86) found no such protection afforded by sitosterol. Similarly, although Rosenman *et al.* (246) found sitosterol administration ineffective in preventing the hypercholesteremia induced in the rat by high cholesterol and cholate feeding, Swell *et al.* (289) found that soybean sterol was a markedly effective hypocholesteremic agent in such circumstances. There is also disagreement in the clinical studies. Some investigators (14, 29, 158) have noted a slight to moderate decrease in the plasma cholesterol of both normo- and hypercholesteremic individuals ingesting sitosterol, but others (111, 309, 285) have not been impressed with the effectiveness of this sterol.

In summarizing these results, it seems fair to conclude that if sitosterol administration does evoke an hypocholesteremic effect, it is very weak even when the sitosterol is given in relatively large doses.

Dihydrocholesterol.—Cholesterol-fed birds were almost completely protected against an expected hyperlipemia, hypercholesteremia, and atherosclerosis when dihydrocholesterol was added to their diet (271). Nichols *et al.* (212) observed a similar protection afforded by dihydrocholesterol to the cholesterol-fed rabbit. Rosenman *et al.* (247) also noted that cholesterol absorption in the rat was inhibited by dihydrocholesterol. Ivy *et al.* (154) and Gould (122) observed that dihydrocholesterol itself was absorbed, and

recently Nichols *et al.* (211) have shown that it acts as an atherogenic substance. On the basis of this last report it seems probable that the possible effectiveness of dihydrocholesterol as a hypocholesteremic agent is due to its absorption competing with that of cholesterol within the intestine. It also seems clear that its practical usefulness as a hypocholesteremic agent is seriously limited by its own atherogenic propensity, a danger to be kept in mind when any therapeutic regimen involving a sterol is considered.

Other possible interfering substances.—Besides sitosterol and dihydrocholesterol, other substances have been described whose ingestion seemingly inhibits the absorption of cholesterol. These are $FeCl_3$ (272), an extract of brain tissue (156), cholesteryl chloride (215), and mineral oil (163).

Course and Character in Intestinal Lymph

All cholesterol absorbed from the intestine is transported via the intestinal lymphatics to the general circulation, a fact demonstrated independently by Biggs *et al.* (31) and by Chaikoff *et al.* (66). The quantitative net increment of such absorbed cholesterol moreover was found by Byers & Friedman (58) to travel in the rat almost entirely in the chylomicron fraction of the lymph, a finding noted in man also by Albrink *et al.* (4).

Since these chylomicra are essentially free of protein (220, 242), being maintained in a dispersed state by their phospholipide content (242), it becomes apparent that the net increment of absorbed cholesterol also exists unbound to protein and thus enters the blood not as a lipoprotein but probably as a lipide solute (4, 58). It thus sharply differs in its physico-chemical status from that increment of cholesterol synthesized and discharged as a soluble lipoprotein by the liver (58).

Plasma Transport and Regulation

The preceding observations suggest that all absorbed cholesterol enters into the subclavian vein in the form of cholesterol-rich chylomicra. This dietary-derived cholesterol continues to circulate in plasma, for a time, as lipide particles (3, 58, 144). This status of dietary cholesterol is evanescent in that it soon disappears from the blood, as does its lipide carrier. To explain the disappearance of this emulsified cholesterol-lipide aggregate, two mechanisms have been proposed. These are the clearing process and the phagocytosis of cholesterol-lipide particles by the hepatic reticulo-endothelial system.

The clearing process.—This process recently has been reviewed by Anfinsen (9). Although Chargaff *et al.* (68) called atttention to the fact that the addition of heparin to plasma produced marked electrophoretic changes with the apparent release of lipide from the β-globulin fraction of plasma, it was Hahn's observation (127) of the clearing of lipemic plasma (i.e., a reduction in the turbidity of plasma) which initiated the numerous studies concerning the clearing or disappearance of lipide particles from dietary or endogenously induced hyperlipemic plasma samples. Although there remains some doubt (see below) about the relationship of this clearing process to cholesterol

metabolism, it seems to the reviewers that this process will be found to be of importance in cholesterol metabolism.

The following phenomena concerned in the clearing process are agreed upon: (*a*) Injection of heparin into a fed animal results in a marked clearing of its post alimentary lipemia, and the plasma of this injected animal is capable of clearing the lipemia of another plasma (6). (*b*) A shift of plasma lipoproteins occurs, proceeding *pari passu* with the clearing process, from those of low density (or high flotation rate) to those of progressively higher density (10, 51, 126, 137, 214). (*c*) rise in plasma free fatty acid also proceeds concomitantly with clearing, apparently resulting from lipolysis of plasma lipides (49, 210). (*d*) An increase in the electrophoretic mobility not only of plasma albumin but also α-and β-lipoproteins is mediated by excess fatty acid (13, 120). (*e*) Plasma albumin acts as the chief but not necessarily the only cofactor in its function of accepting the released fatty acid (51, 120, 317). (*f*) Other substances besides heparin (e.g., dextran and hyaluronidase) may upon injection initate the clearing mechanism both *in vivo* and *in vitro* (49, 131, 264). (*g*) Protamine is capable of reversing or preventing the clearing effect either in heparin-induced or naturally occurring clearing (49, 51, 131, 278).

Although the above data about the clearing mechanism are agreed upon, there are phases concerned with the process which still remain obscure or are points of controversy. For such controversial points, the interested reader is referred to the review of Anfinsen (9).

The hepatic reticulo-endothelial system.—Friedman *et al.* (92, 102, 108, 109) demonstrated by staining, "blocking," and radioactive tracer techniques that in the rat, rabbit, and dog the hepatic reticulo-endothelial cells participate in the normal disposition of exogenously derived, but not of endogenously produced, cholesterol. Apparently the inclusion of exogenously derived cholesterol in chylomicra led to this preferential function on the part of the hepatic reticulo-endothelial cells. In agreement with this concept are the findings of Waddell *et al.* (303) who noted a marked interference with the hepatic deposition of injected lipide particles if "blocking" agents were injected and also those of Rice *et al.* (239) who noted similar interference in hepatic cholesterol deposition in rats fed cholesterol if a blocking agent were administered. However, both Brown *et al.* (50) and Lautsch *et al.* (184) believe that interference with the function of reticulo-endothelial cells also disturbs the endogenous metabolism of cholesterol. Although it now seems clear that the hepatic reticulo-endothelial cells do participate in the disposition of exogenously derived and perhaps endogenously produced cholesterol too, the quantitative significance of their role remains unknown at this time.

The Role of the Liver in the Plasma Regulation of Cholesterol

Removal from blood.—It is possible that small amounts of intestinal absorbed cholesterol are deposited immediately in various tissues. However, as demonstrated in the rat by Friedman *et al.* (60), the overwhelming mass

of absorbed cholesterol is immediately deposited in the liver. Hellman *et al.* (136), employing labeled cholesterol, found that the greater part of absorbed cholesterol quickly disappeared from the blood in man and ostensibly was deposited in the liver.

Homeostasis.—The liver was found both by Friedman *et al.* (106) and Gould (121) to be the chief source of plasma cholesterol, and the indispensable agent not only for the disposal of excess plasma cholesterol occurring from injection (104) but as mentioned above also from ingestion (60). More recently, it has been found (103) that the liver also serves as the source of the cholesterol ester present in plasma.

Fate of exogenously derived cholesterol in the liver.—The ultimate disposition of ingested cholesterol has been investigated by Rosenfeld *et al.* (244). Their results show the end product of absorbed cholesterol to be bile acid.

Excretion and Catabolism of Cholesterol

Excretion.—Less than 20 per cent of the body cholesterol is lost in the feces as cholesterol, coprosterol, or cholestanol (243, 266, 306). While feces from the guinea pig, dog, rat, and man have been found to contain some Δ^7-cholestenol, and guinea pig feces contain 7-dehydrocholesterol, these sterols are quantitatively minor constituents, and in the case of Δ^7-cholestenol possibly may not arise by conversion from cholesterol. Wells *et al.* (306) have found that apparently these sterols can enter the feces with sloughed intestinal mucosa, while Festenstein & Morton (93) have determined that the distribution of 7-dehydrocholesterol in intestine is consistent with the widespread occurrence of a dehydrogenase whose normal substrate is cholesterol. Of course, any ingested sterol may appear in the feces if it has escaped absorption. When interpreting the results of analyses of feces it is also necessary to keep in mind the possibility of microbiological oxidation or even degradation of steroids and splitting of their conjugates (128, 132, 145, 189, 217, 218, 280, 304).

The excretion of cholesterol as such in urine is only a few milligrams per day (165).

Degradation to Bile Acids

Amino acid conjugates of the bile acids are the principal metabolic end-products of blood and liver cholesterol in laboratory mammals and man (1, 18, 22, 23, 37, 55, 82, 87, 90, 104, 117, 130, 177, 192, 267, 269, 270). The steps in the reaction: cholesterol→tauro- (or glyco-) cholic acid and the order of their occurrence is uncertain. However, by combining the suggestions of Lynn, Staple & Gurin (194), Haslewood (130), and Bergström *et al.* (25) a provisional formulation may be attempted as described below. A discussion of this degradation by Siperstein & Chaikoff (266) has also appeared.

Scission of the side chain.—The work of Zabin & Barker (314) in isolating the labeled C-24 of biosynthesized cholic acid has shown that cholesterol is transformed to cholic acid by removal of the terminal isopropyl carbons alone, and not by more extensive degradation of the side chain followed by

resynthesis. Staple & Gurin (283) have come to the same conclusion, as have Bergström *et al.* (25, 28). Lynn *et al.* (194) suggest that the first step in this reaction is conversion of cholesterol to 25-dehydrocholesterol. This unsaturated side chain is then capable of being oxidized (194) to a C-27 carboxyl, C-26 aldehyde ("semi-aldehyde") with introduction of a hydroxyl at C-24. Since C-26 appears in the expired CO_2 both in intact animals (202, 265) and in cell-free systems (8, 97), it is evident that the side chain is decarboxylated and probably further oxidized. Lynn *et al.* suggest that the decarboxylation gives rise to a C-26 aldehyde which forms a β-keto-CoA derivative. In the absence of evidence to the contrary, saturation of the 5, 6 double bond to give *cis* junction between rings A and B may be hypothesized to occur at this point, together with inversion of the 3-β-hydroxyl to give a 3-α group. The C-26 aldehyde would then cleave to lithocholic acid CoA which would conjugate with taurine or glycine.

Oxidation of the nucleus.—A logical progression from cholesterol to cholic acid involves successive hydroxylations of cholanic acid, and this is borne out by the finding of conjugated mono-, di-, and trihydroxycholanic acids in bile (82, 130, 153, 267, 311, 314). Since all the common bile acids and their amino acid conjugates have been prepared as carboxyl-labeled compounds by Bergström *et al.* (24, 26) and have been shown to be readily absorbed, by Sjövall & Akesson (274, 275), it becomes possible to trace metabolic products of each individual bile acid. This is done by administration of labeled bile acid and subsequent isolation and identification of the labeled metabolic products in fistula bile. Results obtained in this way show cholic acid to be one of the final products. For example, Norman (219) administered 24-C^{14} glycocholic acid to rats and recovered almost all the isotope in the bile unchanged, still as glycocholic acid. However, when the less oxidized and unconjugated deoxycholic acid was given to the bile fistula rat by Bergström *et al.* (25) the label was recovered in the bile as taurocholic acid, showing that conjugation and 7α hydroxylation had occurred. These same two reactions also take place in liver slices and homogenates (19, 20, 21) and in cell-free preparations containing formed elements (94, 183, 273). The occurrence of conjugation prior to hydroxylation of the nucleus is suggested by rate studies in the above specialized preparations (19, 20, 21, 25). This same order of reaction is also suggested by the fact that when glycodeoxycholic acid is given (219) glycocholic acid is recovered from the rat instead of the normally excreted taurine conjugate, showing that the glycodeoxycholic acid conjugate was not split before hydroxylation. The conjugation of bile acids by subcellular preparations studied by various workers (21, 46, 47, 48, 118), has been shown by Elliott (91) to involve CoA.

Successive hydroxylation steps preceding the formation of cholic acid are further suggested by the work of Siperstein & Chaikoff (266) who trapped labeled deoxycholic acid in the rat subsequent to administration of cholesterol-C^{14} and unlabeled deoxycholic acid, whereas in the absence of the unlabeled trapping agent the label appeared in cholic acid. Siperstein *et al.*

(267) also observed that bile from rats injected with labeled cholesterol contained no labeled taurocholic acid until after 4 hr. had passed, while these early bile samples did contain taurine conjugates of less hydroxylated bile acids.

Apparently conjugated chenodeoxycholic acid is also a product of cholesterol metabolism in the rat for this acid becomes labeled when cholesterol-4-C^{14} is administered (18, 22, 267). When labeled chenodeoxycholic is administered (27, 196) it is not converted to cholic acid but is recovered in large part unchanged, although it showly becomes converted to two bile acids as yet unidentified. An unknown trihydroxycholanic acid, similar to, but not identical with, cholic acid, was found when labeled lithocholic acid was given to rats (28). Since no radioactive cholesterol (55) or less hydroxylated bile acids (25) are recovered after administration of labeled cholic acid to the rat, there is evidence that the reaction cholesterol→cholic acid is not reversible *in vivo*.

From the above evidence it seems likely that chenodeoxycholic acid, cholic acid, and possibly unknown trihydroxycholanic acids are end products of cholesterol metabolism in the rat. Since human bile also contains cholic and chenodeoxycholic acids, together with lesser amounts of deoxycholic and occasionally lithocholic acid (153, 311), it seems probable that human end products of cholesterol metabolism are similar to those of the rat.

The rabbit differs from the rat in that labeled deoxycholic acid is not transformed to cholic acid (Ekdahl & Sjövall). Since labeled glycodeoxycholic acid, as well as glycocholic acid, is found after administration of labeled cholesterol to rabbits (90), in this species deoxycholic acid must also be a final product of metabolism.

The bile acids are normally lost to the body in the feces, a small fraction escaping enterohepatic circulation at each cycle (157, 268).

Products Other than Bile Acids or Hormones

Cholesterol is converted to 7-dehydrocholesterol (cholesta-5,7-dien-3-ol) by skin, intestine, and other tissues, and this sterol acts as a photochemical precursor of Vitamin D_3 (93, 119, 306). Cholesta-3,5-dien-7-one may also be in this sequence of metabolites since Kantiengar & Morton (162) have shown it to appear in increased quantity in the livers of cholesterol-fed rats and in atherosclerotic human aortas (161). These same authors (160) suggest the metabolic sequence cholesta-3,5-dien-7-one→cholesta-4,6-dien-3-one→cholesta-4-en-3-one→coprostanol. The transformation of cholesterol to coprostanol has been studied in human subjects by Rosenfeld *et al.* (243). Their data suggest direct saturation of the 5,6 double bond.

Conversion to Steroid Hormones

The biosynthesis of steroid hormones has been recently reviewed by Pincus (224) and Staudinger (282) and included by Roberts & Szego (241) in their comprehensive review of steroid biochemistry. In consequence, this

section discusses only work concerning derivation of steroids from cholesterol published between November, 1954 and November, 1955.

Steroids from cholesterol and acetate.—Cholesterol has long been suspected as a precursor or an intermediate in the synthesis of adrenal steroid hormones because of the marked fall in the cholesterol content of the gland following stress. Its actual participation in adrenal synthesis of steroids could be followed more closely when it was found (195, 236, 258) that adrenal homogenates were capable of the complete conversion of cholesterol to corticoids. Bligh *et al.* (33) working with cell-free adrenal preparations also observed utilization of cholesterol for steroid synthesis. They investigated the obligatory role of cholesterol as steroid precursor, but were unable to detect cholesterol in their preparation when acetate was employed as substrate, although, with this exception, similar steroid products were found when either C^{14}-acetate or labeled cholesterol was used as substrate.

Steroidneogenesis from acetate also occurs in ovarian tissue slices or homogenates (233); this synthesis may possibly proceed through cholesterol since labeled cholesterol as well as labeled estradiol and estrone were found.

Although addition of ACTH specifically increases the production of corticoids from cholesterol in whole gland perfusion experiments (241), the rate at which cell-free adrenal homogenates effect the synthesis of corticosteroids from cholesterol does not now appear to be influenced by ACTH (195, 236, 256). It is possible, however, as Macchi & Hechter (195) suggest, that the rate of steroid synthesis by homogenates may be at a physiological maximum, since it is almost three times as rapid as that occurring in adrenal slices.

Cholesterol to pregnenolone.—The steps in the transformation of the cholesterol side chain prior to cleavage at carbon-20 are unknown, although a metabolite tentatively identified as 20-hydroxycholesterol has been found by Lynn *et al.* (194) after incubation of cholesterol with a soluble enzyme system. This enzyme system, prepared from adrenals, testes, or ovaries, also splits cholesterol at C-20, yielding the C_{21} steroid Δ^5-pregnene-3β-ol-20-one (pregnenolone) and caproic acid. The finding of these two products is in accord with the hypothesis that conversion of cholesterol to steroid hormone requires the initial cleavage of the side chain to form a 21-carbon steroid, such as pregnenolone, which retains the β hydroxy Δ^5 configuration of the cholesterol molecule. Pregnenolone has also been detected as a cholesterol metabolite in various other steroidogenic preparations (257, 279).

Pregnenolone to progesterone.—Conversion of pregnenolone to progesterone involves oxidation of the 3-β-OH group and migration of the double bond from ring B to ring A.

The oxidation of the 3-β hydroxy group proceeds by means of a diphosphopyridine nucleotide (DPN) linked sterospecific β-hydroxy dehydrogenase and appears to be reversible (293). The transfer of hydrogen occurs directly between the β-hydroxy and the DPN dehydrogenase without involving any hydrogen from water. This may represent a general mechanism of

steroid hydroxylation since a similar direct hydrogenation also appears to govern the reversible hydroxylation in the 11-position (133).

The migration of the double bond from ring B to ring A is mediated by a steroid isomerase (294) which catalyzes the reactions Δ^5 pregnene,-3,20-dione to α,β-unsaturated ketone, and Δ^5 androstene-3,17-dione to Δ^4 androstene,3,17 dione; but it does not catalyze the isomerization of 5-cholestene-3-one. The particular specificity of this enzyme is in agreement with experimental evidence (286) that cholestenone does not function as a corticosteroid precursor under conditions where corticoids are synthesized by the adrenal. Talalay & Wang (294) show that isomerization proceeds by intramolecular proton transfer, that is, without deuterium incorporation during bond migration.

Progesterone to corticosteroids.—Progesterone appears to be the central precursor (287) in the final elaboration of the known hormonally active C-21 adrenal steroid hormones: 11-deoxycorticosterone (DOC), 17-hydroxy-11-deoxycorticosterone (17-hydroxy DOC), 17-hydroxycorticosterone (F), corticosterone (B), and aldosterone.

Progesterone-21-C^{14} is converted to 17-hydroxy DOC and to DOC by sedimented beef adrenal homogenates (225). By use of this fraction, the hypothesis that C-17 hydroxylation must precede C-21 hydroxylation was reaffirmed. This is shown by the finding that if 17-hydroxy progesterone is added to the incubation mixture no DOC is formed although 17-hydroxy DOC is found, while if DOC is added, no 17-hydroxy DOC is formed. The C-11 hydroxy analogues, B and F, therefore also should not be interconvertible in the adrenal. In another demonstration of this same hypothesis that 17 hydroxylation must precede 21 hydroxylation, progesterone is transformed to B and F by adrenal perfusion, but 17-hydroxyprogesterone goes to F only (186).

Under certain conditions, during the incubation of progesterone (225), in addition to B and F some water-soluble derivatives of progesterone also may be found. These possible water-soluble intermediates have been noted by others (149, 194) and may be members of the class of incompletely characterized steroid compounds containing amino or dipeptide nitrogen described by Voigt & Schroeder (301) which they obtained from aqueous adrenal extracts.

6α, 6β, 18, and 19 hydroxylation.—Three new carbon oxidations have been demonstrated by adrenal homogenate conversion of DOC to 6α or 6β hydroxy DOC and 18-hydroxy to 19-hydroxy DOC (134, 159). 18-Hydroxy DOC appears to be a logical intermediate in the formation of aldosterone. 19-Hydroxy DOC has been isolated from adrenal extract (198) and adrenal perfusate (187) and is of unknown significance. It is inactive in work and salt retention tests. A 19-hydroxy derivative of 17-ketosteroid is formed by adrenal homogenate (203, 205). It too is relatively inactive in bioassays (estrogenic activity unreported) but is transformed by endocrine tissue *in vitro* to estrone (204). This reaction has been suggested as a model

for the biological elimination of C-19 angular methyl group and aromatization of ring A. The transformation of tagged androgen to estrogen has been demonstrated *in vivo* (135) and *in vitro* (12).

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CHEMICAL CONSTITUTION AND IMMUNOLOGICAL SPECIFICITY[1]

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It is now generally recognized that antigens may contain several immunologically reactive groupings or areas. Agreement among workers in the field is nearly unanimous that there are at least two groupings on antibody molecules capable of reacting with antigen. The present discussion is, therefore, based on the theory that the visible immune reactions, such as specific precipitation (the precipitin reaction) and the agglutination of antigenic cells or particles by antibody, are attributable to the combination of antigen, immunologically and therefore also chemically multivalent, with multivalent antibody to yield large aggregates (1, 2). The review is confined to naturally occurring substances and their immediate derivatives.

Proteins

Because a large proportion of antigens consist of, or contain proteins, it would be desirable to relate the antigenic properties of proteins to their chemical constitution. Unfortunately, the present methods of protein chemistry for the determination of fine structure are so time-consuming and laborious that a reasonable degree of certainty exists for only one or two of the simplest derivatives such as insulin, which is not ordinarily an antigen (3). It is possible, however, to record a few pertinent observations. The property of stimulating the production of antibodies in a heterologous host animal seems to increase with increasing molecular weight of proteins, although there is no reason to assume direct proportionality. Whether or not the sulfur-containing residues are in the —S-S— or —SH form is immaterial. This has been shown for four protein-antiprotein systems (4 to 7).

Denaturation of crystalline hen's egg albumin (Ea) results in an almost complete change of specificity. Ea reacts only weakly in rabbit antisera to denatured Ea (DnEa), and DnEa gives only small precipitates in antisera to Ea (8). A simple mechanism by which this might be brought about is shown in Figure 1. In the sketch on the left (A), a portion of the reactive surface of Ea contains an immunologically reactive area, enclosed by the dotted line, and arbitrarily chosen to consist of outcroppings of three amino acids, a, b, and c, which are part of one or more folded peptide chains. This area, together with four or five not necessarily identical areas, gives Ea its characteristic immunological specificity. When Ea is denatured and the peptide chains are presumably extended, amino acids a, b, and c would no

[1] The survey of the literature pertaining to this review was completed in November, 1955.

longer be adjacent components of a single reactive group, but would be separated, as in the diagram on the right (B), and would presumably have different neighbors such as d. This would result in a new specificity, characteristic of DnEa. Some such scheme is in accord with recent studies on the denaturation of Ea and the more reversible denaturation of serum albumin (9). Denaturation of horse serum albumin with urea and reversal of the process by dialysis yields a regenerated product with apparently unaltered specificity (10). Seemingly the peptide chains refold into their original patterns. An important result of the finding that DnEa has a definite and reproducible specificity different from that of Ea is that DnEa cannot be

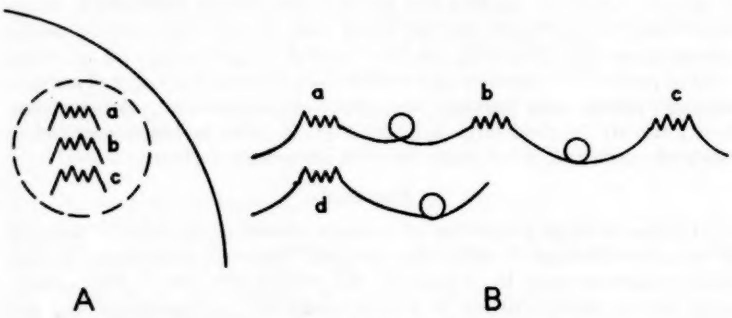


FIG. 1. Diagram illustrating change in specificity as a result of denaturation.

"disordered and disoriented" as has been stated (11), but has definite characteristics which are reflected in the antibodies produced on injection into an animal. This is far from the only instance in which immunochemical experiments have permitted a choice between alternative physical-chemical theories [cf. (12, 13) for instance].

Ea has had other uses as a reagent. It has been found that enzymatic removal of six amino acids to form "plakalbumin" results in a slight change in the curve of precipitation in anti-Ea serum (14). Replacement of one-quarter to one-third of the amino groups of Ea by hydroxyl yields, in part, an undenatured derivative of increased electrophoretic mobility and lowered isoelectric point, but there is no detectable change in immunological specificity, even when the new derivative is used as an antigen in rabbits (6). On the other hand, combination of the amino groups of bovine serum albumin (BSA) with polyglycyl residues containing nearly 200 glycyl units gives rise to a definite, but surprisingly small change in specificity (15).

Consideration has been given to the terminal groupings of peptide chains in proteins as determinants of specificity. Differences have been found in the N-terminal amino acids of the hemoglobins (16) and fibrinogens (17) of different species. These are important indications of species differences in

analogous proteins, but no direct correlation was made with the corresponding immunological specificities, which might well depend upon other molecular groupings. It was not possible to find differences in the N-terminal sequence of four (18) or five (19) amino acids in normal γ-globulin of the rabbit and a variety of antibodies in the same animal. Evidence regarding the C-terminal end of the protein chain is not entirely negative. Although carboxypeptidase splits off two molecules of alanine, one of leucine, and one of threonine per molecule of BSA, the residual product gave the same quantitative precipitin curve with antibodies to BSA as did the native albumin (20). On the other hand, removal of threonine alone from tobacco mosaic virus, V, by carboxypeptidase causes a definite change in specificity (21). Although much cross-reactivity remains, V does not precipitate all of the antibody in antiserum to the enzyme-treated-V, nor does the latter precipitate all of the antibody in antiserum to V. A change in specificity is therefore indicated. Although almost the same proportion of the molecule is removed in the case of both BSA and V, the disparity in molecular weights is so great that the number of residues removed is as 4:2900. Possibly, then, the change in antigenic properties of V is attributable to the enormous number of repetitions of the small difference. Multiple reactive groupings of a somewhat different specificity could be made available in this fashion for precipitation with antibody.

Since antibodies are modified serum globulins it is pertinent to discuss at this point two recent papers dealing with the dimensions of the sites on the antibody molecule which combine with antigens. In confirmation and extension of numerous studies indicating that ions often take part in this union, it has been concluded that a single carboxyl group is involved in the bond linking BSA and rabbit anti-BSA (22) and that a corresponding positive charge is involved in each complementary site. Although this may be true in this system, there is no evidence that the properties, with respect to changes in pH (22), of specific precipitates between antibodies and uncharged polysaccharides are any different from those of protein-antiprotein systems. Possible implication of a single carboxyl group is also not necessarily "a reflection of the relatively small size of a reactive site on these large molecules," as a glance at Figure 1, for example, will readily show. A single carboxyl group may be only an essential detail in a relatively large determinant area. That such an area is not small is once more indicated by studies on the dimensions of the combining sites in the dextran human antidextran system (23). Oligosaccharides containing three to five glucose residues in the proper linkage were found to inhibit specific precipitation far more efficiently than smaller or larger chains, indicating an optimal fit with antibody at possibly four glucose residues. Complementary to this would necessarily be a fairly large area of antibody protein surface.

A capsular D(-)polyglutamic acid reactive with antibodies and first isolated from *Bacillus anthracis* and later from the *B. subtilis* and *mesentericus* groups, has been shown to be mainly a γ-polypeptide (24).

Polysaccharides

Second only to the proteins in their variety are the polysaccharides, so that it is not surprising that they are often marked by immunological specificity. Moreover, the art of the determination of fine structure in carbohydrate chemistry is more advanced than in the case of the proteins, and somewhat less time, effort, and patience are required for disclosure of relationships between structure and specificity. Nevertheless, there have been comparatively few such studies since the discovery that type-specificity among many encapsulated microorganisms was a result of their outer layer of polysaccharides which are resistant to the action of the common carbohydrate-splitting enzymes and different in composition for each bacterial type (25). Among the 70-odd serological types of Pneumococcus alone, the component sugars of the capsular polysaccharides are known for only a few (Table I), and the fine structure of only one has been worked out (26). Even in this instance (Type III) it is not certain whether the linkage between the cellobiuronic acid units, of which the substance is a polymer, is in the α- or β-configuration. This much knowledge sufficed, however, to account

TABLE I

Specific Polysaccharides of Pneumococci and the Tubercle Bacillus

Type	$[\alpha]_D$ degrees	N per cent	Acetyl per cent	Component sugars
		Pneumococci		
I	+275	4.6	7.1*	Galacturonic acid, unknown constituents
II	+55	0.2	<1	L-rhamnose, D-glucose, D-glucuronic acid
III	−35	0.05	0.5	1→3 polymer of cellobiuronic acid
IV	+30	5.5	5.8†	Unknown
VIII	+123	0.2	0.5	Cellobiuronic acid, D-glucose, D-galactose
IX	+117	3.1		Glucose, amino sugar, uronic acid
XII	−1	4.1		Galactose, glucose, amino sugar
XIV	*Ca.* +10	2.8		D-galactose, N-acetylglucosamine, glucose
XVIII	+88	0.3		L-rhamnose, D-glucose, sec. bound PO_4
		Mycobacteria		
Tubercle bacillus, human type				
B fraction	+27	0.8		D-Arabinose, D-galactose, D-mannose, D-glucosamine
C fraction	+85	0.1		D-Arabinose, D-mannose, L-rhamnose

* O-acetyl.
† N-acetyl.

for a long-known serological relationship, or cross reaction, between Types III and VIII pneumococcus (27). The specific polysaccharides of both types were shown to contain the same aldobiuronic acid unit (28), later identified as cellobiuronic acid. The same cross reaction was then studied quantitatively and interpreted (29) in terms of the quantitative theory of the precipitin reaction (2). With the help of this theory it could be predicted that oxidized cotton, which contains multiple units of cellobiuronic acid, would be an immunologically specific polysaccharide reactive in Type III and Type VIII antipneumococcus sera, and the prediction was readily verified (30).

It will now be shown that the theory serves as a reliable guide in the search for other correlations between constitution and specificity. Through the study of cross reactions of polysaccharides of known constitution, the theory has provided short cuts and clues to the structure of some of the immunologically specific bacterial polysaccharides—information otherwise difficult of access and henceforth more easily obtainable because of these preliminary indications. Moreover, the immunochemical methods employed have proven to be powerful tools for the identification of unknown components in polysaccharides under investigation, and for determination of the homogeneity of carbohydrates. The latter use has already been emphasized in the case of the blood group substances (31). Another striking finding is that every polysaccharide is found to be potentially an immunologically specific one, insofar as it contains some of the repeating structural units common to the microbial polysaccharides which, in the intact microbial cell, or in the isolated state, stimulate the production of antibodies in animals.

The polyglucoses.—Under this heading are discussed many polysaccharides that owe their serological reactivity to multiple recurrences of an appropriate linkage of glucose.

Many years ago it was found that the dextrans which were synthesized by various strains of *Leuconostoc* and other microorganisms gave cross reactions in Types II, XII, XX, and XXII antipneumococcus sera and antityphoid sera (32). More recently the use of dextran as a plasma expander has aroused much interest, but it is probable that some of the accidents which have resulted from the injection of dextran are attributable either to preexisting antibodies to dextran itself, for dextran is antigenic in man (33), or to cross reactions with other antibodies, because the polyglucose group of specificities is very widespread and is found among many of the *Salmonella* microorganisms as well as among pneumococci. Even synthetic polyglucoses, made by heating glucose with a mild catalyst, may show these specificities. The synthetic polyglucoses are mixtures with more or less random linkages of glucose, but in which 1,4-, 1,6-, and 1,4,6- linkages predominate (34). Before the introduction of any such substance into use as a plasma expander, it was desirable to test its specificity at least against the same antisera that were reactive with dextran (35). As may be noted from Table II, a typical

synthetic polyglucose showed almost identical reactions with those of a typical dextran. It could also be fractionated in such a way as to yield fractions which either precipitated up to 50 per cent of the total antibody in types XII and XX antisera, or separated into portions of presumably lower molecular weight or different chemical linkage or both, which precipitated very little antibody.

At this time Stacey stated that all of the glucose in the specific polysaccharide of Type II pneumococcus was in the form of 1,4,6- branch points (36). This observation, considered in the light of the quantitative theory of the precipitin reaction, leads to the deduction that any polysaccharide containing multiple 1,4,6- branch points of glucose should react with Type II antiserum and, perhaps, show some of the other precipitin reactions characteristic of dextrans which contain 1,4,6- branch points, and of the synthetic polyglucoses which also have such linkages.

The first polysaccharide that one thinks of with 1,4,6- branch points of glucose is, of course, glycogen. But, according to the old-fashioned immunology, since glycogen is everywhere in the animal body, it could not be expected to react with any of these antisera. However, as noted in Table II, the prediction was readily verified, for a number of glycogens of widely different distribution reacted very heavily in many of these antisera (37). It is glycogen which precipitates, because if one spits into it the reactivity with antisera disappears, and also because even glycogen from horse liver and horse muscle reacts as heavily in the antipneumococcus horse sera as do glycogens from other species. This could not happen if a protein impurity were responsible, since homologous proteins are not antigenic. The precipitation is attributable to combination of the glycogen with type-specific antibody, for if one adds the amount of nitrogen, often considerable, precipitated by the glycogen, to the amount obtained from the supernatant fluid on addition of the type-specific polysaccharide, the sum equals the total precipitated from an equal quantity of antiserum by the type-specific polysaccharide alone.

To a somewhat less extent, the same is true of amylopectin (Table II). Precipitation is usually weaker, presumably because the 1,4,6- branch points of amylopectin are farther apart than in glycogen.

Another polysaccharide which reacted as predicted was that of tamarind seed (jellose). It is made up of xylose, galactose, and glucose, two-thirds of the glucose residues being linked in the 1,4,6- positions and the remainder at 1,4- (38). Some of its cross reactions are given in Table II. Like glycogen, it precipitated 2 per cent of the antibodies in a Type II antipneumococcus horse serum.

Although some of the dextrans which react as in Table II undoubtedly contain 1,4,6- branch points of glucose, dextran N 236 was chosen for the Table because oxidation with periodate shows it to have 96 per cent 1,6- linkages and 4 per cent of 1,3- or 1,2- bonds. It is, therefore, quite possible that multiple 1,6- linkages, perhaps in chains, are as effective as scattered

TABLE II

QUALITATIVE TESTS WITH POLYGLUCOSES AND ANTIPNEUMOCOCCAL HORSE SERA AT 0° C.*

Serum, type:	II	III C†	VII	IX	XI	XII	XVIII	XX	XXII	XXVII	XXVIII	Normal horse sera		
												a	*b*	*c*
Dextran, N236	+++		++	++	−	+++	+++	+++	+++					
Synth polyglucose, 52R2109.	+++	+±	+++	+++	+±	+++	++	++±	++++					
Source of glycogen														
Cat liver	++		+±	+++	±	+++	−	++±	+++			−		
Dog liver	+±		−	+++±	+	+++		++±	+++±	−		+	++	++
Human, v.G.1.	+±		+±	+++	±	++±	±	++±	+++±	−	±	−	++	++±
Rabbit liver	++		+	++±		+++		++±	+++					
Horse muscle	+	−	−	+++	±	+++	−	++±	+++				++	
Horse liver, acid prepared	++−	−	+	+++	+±	+++±	+	+++	+++					
Horse liver, alkali-prepared	+++	−	++	+++	++	+++±	++	+++	+++				+++	
Oyster	++±†			++±		++±		++±	+++	−	+		++	++±
Maize	++		++	++±		++±	+	++	+++					
Phosphorylase limit dextrin	++		+±	+++	+	+++	±	+++	++±			±		
β-amylase limit dextrin	+++±		+±	++±	±	+++±	±	+++	++±			−		
Maize amylopectins														
Alkali-prepared	+†		+	+±	±	+, +++‡	±	+±	++±			+		
Acid-prepared	±†		+	+	+	±, ±‡	±	+	+			+		
β-amylase limit dextrin	+		±			++±, ++‡	−	++	++					
Tamarind seed polysaccharide	++			+±	++	++		++±	+++					

"Normal" horse serum *b* gave ++ reactions with the specific polysaccharides of Types II and XII pneumococcus; serum *c* reacted only with the latter.

* Reprinted and amended from Reference 37.

† Serum absorbed with pneumococcal C substance, the somatic, group specific polysaccharide.

‡ Two different antisera.

1,4,6- branch points in the cross reaction. This is possibly indicated as well by the results of quantitative precipitin estimations at 0°C. (37). Oyster glycogen precipitated only 2 per cent of the antibody in a Type II antipneumococcus horse serum, but carried down 16 per cent from Types IX, XII, and XXII antisera. In the latter instances the 1,4,6- linkages of the glycogen are not necessarily involved; possibly multiple 1,6- or 1,4- linkages or both determine cross reactivity in these sera. E. A. Kabat (personal communication) suggests that multiple end groups of single glucose residues might be involved in all cross reacting sera.

Because of the cross reactions and the availability of highly purified samples of the specific polysaccharides of Types IX and XII pneumococcus, these carbohydrates were hydrolyzed and the solutions chromatographed (39). As might have been predicted from the cross reactions of Types IX and XII antisera with polyglucoses, both substances contained glucose, although this prediction was rendered unsafe by lack of information as to whether or not glucosamine differs from glucose in its specificity. Both polysaccharides also contained amino sugars, so that much more work will have to be done before the origin of the cross reactivity of the two antisera is made certain. The precipitation caused by the polyglucoses in Type XVIII antiserum is also presumably attributable to antibodies stimulated as a result of the glucose content of the capsular polysaccharide of Type XVIII pneumococcus, although the glucose units must be in very different linkage from those in the Type II substance since there is no cross reactivity in either direction between Type II and Type XVIII pneumococcus (40).

Specificities attributable to multiple recurrences of galactose.—Until 1954 only the specific polysaccharide of Type XIV pneumococcus was known to contain galactose (41). Other constituents are N-acetylglucosamine (41) and glucose (39). Isolation of a galactan from the residues of beef lungs after the separation of heparin (42) provided the occasion for testing the galactan for cross reactivity in Type XIV antipneumococcus horse serum. Although three kinds of galactose linkages at positions 1-, 1,6-, and 1,3,6-, probably all β-, had been found in the lung galactan (43), precipitation of Type XIV antibodies was not predictable since the mode of linking of the galactose in the Type XIV polysaccharide was unknown. As it happened, 28 per cent of the antibodies in the antiserum were precipitated (44). A clue as to which linkages of the galactan were implicated was provided by the somewhat weaker precipitation given by tamarind seed polysaccharide (jellose), which had also been of interest as a polyglucose. In jellose all of the galactose occurs as nonreducing end groups (38), one of the three forms of the sugar in the lung galactan. Although the Type XIV pneumococcus polysaccharide, like jellose, contains glucose as well as galactose, it is unlikely that jellose reacts with antibodies in Type XIV antiserum directed against multiple recurrences of glucose, since lung galactan, which contains no glucose, removes almost all antibodies reactive with jellose (44). One could therefore predict that other polysaccharides containing nonreducing end groups of

galactose would show similar reactivity. Indeed, the next located, carob bean mucilage, a galactomannan in which all of the galactose fulfills this condition, precipitated the Type XIV antiserum more strongly than did jellose (45). A further prediction may therefore confidently be made; namely, that when the fine structure of the Type XIV pneumococcus polysaccharide is worked out, a portion, at least, of its galactose residues will be found to occur as nonreducing end groups.

Certain other galactose-containing polysaccharides also precipitate in Type XIV antipneumococcus serum (45). One of the most potent is the arabogalactan of the Jeffrey pine which precipitates practically as much antibody as does lung galactan. It has, however, only one galactose end group for every 28 sugar units, and most of its linkages of galactose are 1,6- and 1,3,6- (46), much like those of the interior bonds of the units in lung galactan. It would therefore appear that such linkages also suffice as determinants of cross reactivity in the Type XIV antiserum. Equally reactive are many preparations of the blood group A substance, but these will be discussed in a separate section.

In the order of decreasing precipitating power in Type XIV antiserum are the following gums, in which galactose is the only sugar in common with the specific polysaccharide of Type XIV pneumococcus: (*a*) the arabogalactan of the Jeffrey pine; (*b*) the arabogalactan of the larch, which contains more galactose end groups (47) than (*a*); (*c*) gum arabic (48); (*d*) the gum of *Acacia pycnantha* (49); (*e*) the galactan of *Strychnos nux vomica* (50); and (*f*) karaya gum (51). Removal of arabofuranose from *a*, *b*, and *c* by partial hydrolysis scarcely affects the quantity of antibody precipitated.

In *e* there are as few end groups as in *a*; in *f* two-thirds of the galactose is in the form of nonreducing end groups, and the rest are linked mainly 1,4-, while in *e* there are also 1,4- bonds and a few 1,3,6- links. Since uronic acids are known to have a strongly directive effect on immunological specificity (52), it is possible that the glucuronic acid in *c* and *d* and the galacturonic acid in *f*, by altering the specificity in a different direction, prevent the galactose in these gums from having as great an influence as might have been expected. Data on the polygalactoses are summarized in Tables III and IV.

Two by-products of the study of lung galactan demonstrate the wide applicability and power of immunochemical methods. It was at first embarrassing to find that the galactan precipitated two-thirds as much antibody from a Type II antipneumococcus serum as from the Type XIV serum, because the specific polysaccharide of Type II pneumococcus is known to contain only rhamnose, glucose, and glucuronic acid (53, 54). However, more careful perusal of Wolfrom *et al.* (43) disclosed that an unknown uronic acid was present to the extent of one residue for every 35 to 40 galactose units. Precipitation in the Type II antiserum could be explained only if the unknown acid were glucuronic acid. Under this stimulus renewed chemical studies were made, and the presence of glucuronic acid was confirmed (44). Moreover, an electrophoretic diagram had shown the galactan to be inhomo-

TABLE III

PRECIPITATION OF 1.0 ML. TYPE XIV ANTIPNEUMOCOCCUS HORSE SERUM 635 BY ARABOGALACTANS AND RELATED GUMS AT 0°C.*

The antiserum contained 47 μg. anti-C nitrogen and 920 μg. anti-S XIV nitrogen per ml. Volumes actually used ranged from 1.0 ml. 1→2 dilution to 2.0 ml. undiluted serum, but all results are calculated to 1.0 ml. "C-absorbed" serum refers to serum deprived of its antibody to C-substance, the somatic polysaccharide of pneumococcus.

Amount of polysaccharide used, mg.	Antibody nitrogen (μg.) precipitated by							
	Gum arabic		Gum, *Acacia pycnantha*		Arabogalactan, Jeffrey pine		Arabogalactan, larch	
	Intact	Partially hydrolyzed	Whole ser.	C-absd.	Intact	Partially hydrolyzed	Intact	Partially hydrolyzed[e]
0.05	30							
.1		32[b]	21			80		46
.2					108		64	
.3		35	30			126		
.5	45			28				
.6					173		85	72
1	43		35			171		83
2				30	218	181	91	
2.5	37[a]					191		
3		9			245		92	
5						173		
6		2			206[c]		90[d]	
10		2			187	138		

[a] Supernatants of all tubes combined: with pine arabogalactan, 202 μg. N; the supernatant from this, with S XIV, gave 805 μg. N, total 1046. Gum arabic precipitated 41 μg. N from C-absorbed serum.

[b] C-absorbed serum gave 29 μg. N, the maximum, at this level. The supernatants with 2 mg. of larch galactan, gave 73 μg. N; with 0.6 mg. of partly hydrolyzed galactan, 67 μg. N, with 1 mg., 74 μg. N.

[c] Combined supernatants from these and preceding tubes, mean N already pptd., 176 μg.; with S XIV, 774 μg. N; with lung galactan, 26 μg. N, followed by S XIV 713 μg., total 915.

[d] Combined supernatants with pine arabogalactan, 113 μg. N; supernatant from this with S XIV, 779 μg. N, total 975.

[e] Analyses with C-absorbed serum.

* Reprinted from reference 45.

TABLE IV

PRECIPITATION OF 1.0 ML. TYPE XIV ANTIPNEUMOCOCCUS HORSE SERUM 635 BY VARIOUS GALACTOSE-CONTAINING POLYSACCHARIDES AT 0°C.*

Amount of polysaccharide used, mg.	Antibody nitrogen (μg.) precipitated by					
	Human blood group A subst., data from ref. 72a	Lung galactan	Jellose	Galactan *Strychnos nux vomica*	Karaya gum	Carob mucilage
0.015				2	17	
.04					20[d]	
.1	66	103	86		18	126
.3	134	179		8		15[d]
.4			94[b]			153[e]
.5	178				14	
.6				12		159[e]
1	236	257[a]	77		8	
2			54	27[c]		
5				31		
6		181	2			

[a] Supernatant plus jellose gave 2 μg. N; + pine arabogalactan, 24 μg. N; + S XIV, 666 μg. N, total, 923 μg.

[b] Supernatant plus lung galactan gave 148 μg. N; + karaya, 16 μg. N, after which pine arabogalactan gave 162 μg. N; a supernatant from karaya and jellose in this order gave 159 μg. N with the arabogalactan. Two supernatants from absorptions with jellose, karaya and pine gave 750, 739 μg. N with S XIV, total N, 1028, 1007. Evidently when the three gums are present together, up to 10% of non-specific N may be carried down. C-absorbed serum and 0.3 mg. jellose gave 89 μg. N.

[c] From C-absorbed serum.

[d] Supernatant + jellose gave 93 μg. N, after which lung galactan precipitated 152 μg. N, calcd. 149. Karaya supernatants + gum arabic gave 27 μg. N; + partially hydrolyzed gum arabic, 11 μg. N; + galactan from *Strychnos nux vomica*, 7 μg. Antiserum absorbed with C substance gave 19 μg. with karaya gum.

[e] Supernatants combined; +S XIV, 784 μg. N, total 940.

* Reprinted from reference 45.

geneous (43). Immunochemical methods gave more detailed information, showing that a fraction, richer in glucuronic acid, could be separated from the principal galactan. This was accomplished by precipitation of separate portions of the galactan with Type II and Type XIV antipneumococcus sera. In the first of these, precipitation is presumably mediated by multiple

recurrences of glucuronic acid; in the second, by multiple groupings of galactose. Extraction of the washed specific precipitates by trichloroacetic acid yields their carbohydrate components quantitatively. If the ratios of glucuronic acid to galactose are the same in the two precipitates as in the galactan itself, the substance is either homogeneous or it has not been fractionated by the treatment (for an instance of the latter, see below); if the ratios are different, the material is a mixture. Actually, the glucuronic acid: galactose ratio in the Type II precipitate was five times that in the Type XIV complex and twice that in the original product (44).

Partial specificities of a type specific polysaccharide.—It has been possible, by means of cross reactions, to separate the specificity of Type II pneumococcus into three partial specificities, each characteristic of one of the three component sugars or sugar acid, L-rhamnose, D-glucose, and D-glucuronic acid (55). One of the earliest known cross reactions of antipneumococcus sera was the precipitation of Type II antiserum by gum arabic in low dilutions (56). Removal of a portion of the pentose of the gum with cold acid resulted in reactivity at several hundredfold higher dilutions. While it was apparent that in gum arabic, some structure common to the specific polysaccharide of Type II pneumococcus had been laid bare by the partial hydrolysis, a detailed explanation of the reaction and of the differences in titer was not possible until quantitative analytical micromethods (2, 57) and more details of the structure of the two polysaccharides (36, 48) became available. Quantitative analyses at once answered the riddle of the titers, since very much larger amounts of native gum than of degraded gum were required to precipitate appreciable quantities of antibody. Although the antibody which could be forced to precipitate by very large amounts of native gum was twice as great as at the maximum with degraded gum, precipitation with the latter required several hundred times less substance. The titers, then, merely reflect differences in combining proportions and "avidity" of each reaction and are useless for the quantitative comparison of either the antigens or the amounts of reactive antibody. In the native gum many of the arabofuranose units are attached to glucuronic acid residues (48), and these pentose molecules evidently block access of the antibody to the multiple sugar acid groupings on the principal chain or chains of the gum. Degradation of the gum by acid not only removes these hindrances and some rhamnose as well (55), but at the same time converts some of the glucuronic acid residues to end groups, a characteristic in common with the Type II polysaccharide (36). The possible importance of end groups for reactions between antigen and antibody has recently been emphasized (23). It is, therefore, understandable why a far smaller excess of degraded gum is necessary to bring the reaction with antibodies to completion. The vague immunological concept of "avidity" is replaced in this instance by a definite chemical picture. The native gum has a sedimentation constant of about 9 in the ultracentrifuge, while the degraded gum shows only 1.6, indicating that some of the main sugar linkages are split in addition to arabofuranose residues.

This probably explains why the total amount of antibody precipitated by the degraded gum is less than that thrown down by the native gum.

The gum of *Acacia pycnantha*, which contains about one-third as much glucuronic acid as does gum arabic, precipitates less than one-third as much antibody from the Type II antiserum. Possibly the distribution of the few residues is unfavorable, although if this is the explanation, the distribution of the even fewer glucuronic acid residues in lung galactan must be nearly ideal, for it precipitates nearly as much antibody as does degraded gum arabic.

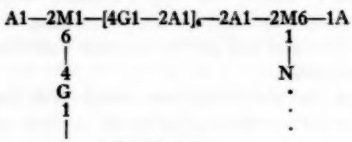
These studies also confirmed the fractionation of gum arabic by precipitation with Type II antipneumococcus serum. It had been stated that such precipitates contain no rhamnose (54), a usual constituent of the native gum, but in the newer work, carried on with larger samples, rhamnose was found, usually one-third to one-fifth of that present in the gum used (55). This indicated that a polyaraborhamnogalactoglucuronic acid of much lower rhamnose content is separated from the native gum by reaction, through multiple glucuronic acid residues, with Type II antipneumococcus serum. That the remaining material is also a polyaraborhamnogalactoglucuronic acid is indicated by the failure of Type XIV antiserum, with which reactivity is attributable to some of the repeating galactose groupings, to effect a similar separation (45). A conclusion of chemical and possibly industrial interest, arrived at by immunochemical means, is that gum arabic is a mixture of closely related gums of differing rhamnose content, and that its composition is therefore uncertain and consequently also some of the chemical structures involved. Tempting speculations would be that the portion precipitable by Type II antiserum might contain more glucuronic acid end groups, whether or not blocked by arabofuranose, or more suitably spaced combinations of rhamnose and glucuronic acid units than are contained in the unprecipitated portion.

In addition to the carbohydrates which give precipitates in Type II antipneumococcus serum because of their content of multiple residues of suitably linked glucuronic acid or glucose, there are others in which rhamnose furnishes the only chemical basis for reactivity. Examples are as follows: (*a*) karaya gum (51), a complex polysaccharide containing rhamnose, galactose, and galacturonic acid; (*b*) the polysaccharide of high rotation from the tubercle bacillus (58, 59) which contains rhamnose, galactose, and D-arabinose; and (*c*) the C-substance, or group-specific polysaccharide of Group A hemolytic streptococcus, composed of rhamnose and glucosamine (60). These substances show reactivity of the same order of magnitude as that of glycogen and jellose.

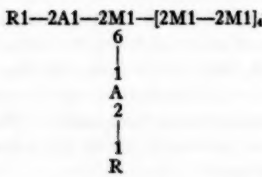
Since roughly 40 per cent of the antibody in one of the Type II antisera and 16 per cent in the other were precipitated by products containing multiple units of glucuronic acid, and much smaller amounts were precipitated by the carbohydrates containing only glucose or rhamnose, it is evident that the sugar acid is the chief determinant of the specificity of Type II pneumo-

coccus. It must be remembered, however, that in these two sera, 50 and 80 per cent of the antibodies, respectively, do not precipitate with any of the heterologous substances tested, and that even the cross-reactive fraction of the antibodies reacts more firmly, and in entirely different proportions, with the capsular polysaccharide of Type II pneumococcus. The antibodies in the serum are, then, directed against multiples of a rhamnoglucoglucuronic acid, and the sum of the three partial specificities falls far short of equalling the whole.

Structure and specificity of polysaccharides of the tubercle bacillus.—Polysaccharides of two different specificities have been recognized in the tubercle bacillus and in tuberculin: one, Fraction B, low-rotating and containing an appreciable percentage of nitrogen, the other, fraction C, of higher rotation and essentially nitrogen-free (58). Methylation of apparently these same two substances has led to the following as possible but not exclusive structures for each (59), in which A, G, M, N, and R represent respectively D-arabofuranose, D-galactopyranose, D-mannopyranose, D-glucosamine, and L-rhamnopyranose: the low-rotating, or lipide bound, polysaccharide is represented by structure I; the high rotating, or somatic, polysaccharide is represented by structure II. Glucosamine was inferred to be present (59) but not isolated from the latter substance. However, it cannot be a part of the molecule, since the polysaccharide has been obtained almost nitrogen-free (58). The differences in specificity are presumably a result of the occurrence of arabinose end groups and multiple units of galactose and glucosamine in the one polysaccharide and rhamnose end groups, with no galactose or glucosamine in the other.



Structure I



Structure II

The blood group substances.—For many years the chemical reasons for the immunological differences of the blood groups remained obscure, for the over-all composition and the component sugars of the numerous substances

with A, B, O, or other specificity seemed nearly the same. Recently, however, differences have been shown.

Among the important factors in the partial clarification of a very complicated situation were, (*a*) discovery of the cross reaction between blood group A substances and Type XIV antipneumococcus horse serum (61) and the cross reaction of some of the B and O substances in the same antiserum (62), (*b*) application of quantitative micromethods (63) to the estimation of antibodies to the blood group substances and to the composition of the specific precipitates (64), (*c*) discovery of fucose in several of the substances (65, 66), (*d*) recognition that both glucosamine and galactosamine are components (67), and (*e*) isolation of material from a single individual (68, 69), although even in such instances the findings may depend upon the subject's homo- or heterozygosity with respect to the blood groups. There remains, also, an uncertainty as to the relation of the isolated, purified materials to the substances as they exist in the animal body. Unless obtained by very mild procedures (70) the blood group substances, which are usually isolated from peptic digests by fractionation with phenol or by phenol treatment of cyst fluids, are not ordinarily antigenic in rabbits. The part played by amino acids in the specificities of the blood groups substances is also not understood.

A few fairly definite statements may be made, however, regarding the carbohydrate portion. Use of a quantitative method for the separate estimation of glucosamine and galactosamine showed that hog and human A substances contained these sugars in the ratios 1.5, 1.6, while the O(H) substances from the same species showed ratios of 2.2 and 2.5; the ratio for the human B substance was 2.8 (71). On mild acid hydrolysis of A substance the A activity was reduced or lost while the cross reactivity with Type XIV antiserum increased (72). A similar but less regular effect was observed with B and O substances. At the same time many of the fucose end groups (65) were split off, as well as some of the amino sugars and galactose, in free and combined form. Studied in greater detail, this reaction showed that a non-dialyzable residue remained with lower ratios of glucosamine to galactosamine than in the original materials (73). The dialysate contained both amino sugars, except in the case of the human B substance, from which only glucosamine was split. Accordingly, there appears to be, in the different substances, a main chain of galactose and N-acetylamino sugar residues closely related to the capsular polysaccharide of Type XIV pneumococcus (61), with acid-labile oligosaccharide side chains of fucose and different ratios of the two amino sugars. Similar views have emerged from an entirely different approach; namely, a study of the decomposition of the blood group substances by enzymes and the inhibition of the degradation by separate addition of the various sugar components (74).

Conclusion

It should now be apparent that the study of the relation of chemical constitution to immunological specificity is still in its early stages so far as

naturally occurring substances are concerned. The immunochemist, of course, depends upon the existing state of the art of the chemistry of proteins and of carbohydrates for his knowledge of how to trace the relationships in these two classes of materials which are so fundamental to the ultimate understanding of the mechanisms of immunity to infectious disease. The immunochemist cannot, however, escape from a certain measure of blame, if anyone is to blame, for the present unsatisfactory situation with respect to the exceedingly complicated substances and organisms which he studies. After all, he is a chemist, and if available chemical methods are too laborious, too tedious, and too equivocal, it is up to him to devise better ones. The writer hopes that by holding up a mirror and reflecting certain highlights and shadows of his subject he may succeed in stimulating a few photoreceptors in himself and in others.

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METABOLISM OF DRUGS AND OTHER ORGANIC CHEMICALS[1,2]

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A change in title of a given subject in *Annual Reviews* is usually indicative of an evolving viewpoint and of more precise definition of biochemical interest. Thus, in 1948 the title, "Detoxication Mechanisms" gave way to "The Metabolism of Drugs and Toxic Substances," in the review by Bodansky (19). Williams (197) considered this title inaccurate since the discussion usually centers around inert organic chemicals which lack unusual toxicity. "Toxic substances" is an all-inclusive term, which may apply equally to the elements such as lead or arsenic or to complex molecules, such as bacterial toxins. The substitution of "organic chemicals" for "toxic substances" in the present title is intended to bring to mind the usual laboratory organic chemicals, whose fate in the animal body has been extensively studied. Reports of such investigations have been the traditional material for annual review in these volumes.

The predictions of Williams in 1951 have been correct with regard to advances which were expected to follow the application of newer techniques, such as absorption spectrophotometry, partition and paper chromatography, labeling with tracer elements, counter-current extraction, and the employment of preparations of β-glucuronidase and sulfatase for identifying metabolic products.

In addition to the progress thus made in research on older problems, newer fields of investigation have been opened, in the search for and characterization of enzymatic mechanisms concerned in metabolic conjugations, chemical synthesis of glucosiduronic acids, penultimate ($\omega-1$) oxidation of alkyl radicals, perhydroxylation of aromatic rings, the metabolic fate of organic chemicals in insects, and the biochemical site of action of certain drugs.

With regard to nomenclature and usage of terms many investigators now use the adjective "metabolic" and others, "biological" in referring to processes of reduction, methylation, conjugation, etc., which organic chemicals and drugs undergo *in vivo*. Glucosiduronic acid is the officially recom-

[1] The survey of the literature pertaining to this review was completed in September, 1955.

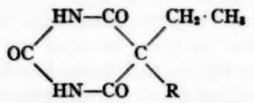
[2] The following abbreviations are used in this chapter: DPN for diphosphopyridine nucleotide; INH for isonicotinic acid hydrazide; TPN for triphosphopyridine nucleotide; TPNH for triphosphopyridine nucleotide (reduced form); UDP for uridine diphosphate; UDPG for uridine diphosphate glucose; UDPGA for the glucuronic acid analogue.

mended chemical term for describing the oside of glucuronic acid, being synonymous with the widely used "glucuronoside" and "glucuronide."

It appears logical to deal with the metabolism of individual drugs and organic chemicals first and then to evaluate the information they contribute to general mechanisms of metabolism. The implications of certain recent developments are then discussed (p. 675) under the section entitled Comments.

Metabolism of Certain Drugs

Dialkylbarbiturates.—Maynert and his co-workers [Maynert & Dawson (123); Maynert, Dawson & Washburn (124, 125)] have isolated and characterized from dog urine metabolites of pentobarbital, amobarbital (Amytal), and butethal (Neonal) of distinct biochemical interest. These substances represent new examples of penultimate ($\omega-1$) oxidation (see structures Ia, b, c). Hydroxypentobarbital was identified also in human urine by Brodie *et al.* (37).



Ia. Hydroxypentobarbital R=—$CH(CH_3)\cdot CH_2\cdot CHOH\cdot CH_3$
Ib. Hydroxyamytal R=—$CH_2\cdot CH_2\cdot COH\cdot (CH_3)_2$
Ic. Hydroxyneonal R=—$CH_2\cdot CH_2\cdot CHOH\cdot CH_3$

Maynert (122) has reviewed the question of whether these products should be regarded as examples of γ- or $\omega-1$ oxidation. On the basis of a study of the metabolites of hexethal (Ortal) which contains a six carbon alkyl chain, he favors the $\omega-1$ carbon atom as a specific site of oxidation of alkyl-radicals in the barbiturates. The hydroxylated metabolites of pentobarbital were considered to be glucuronic acid conjugates on the basis of strong indirect evidence. It is intriguing that the 1-methylbutyl group when attached to a thiobarbituric acid ring (thiopental) undergoes ω- rather than $\omega-1$ oxidation [Brodie *et al.* (38)]. Perhaps the localization of thiopental in body fat may be important in this connection [Brodie, Bernstein & Mark (36)]. Maynert sounds a note of caution on the basis of this comparison (pentobarbital versus thiopental) regarding the dangers of reasoning by analogy in problems of metabolic fate.

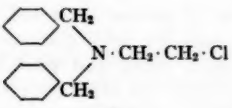
An interesting approach to the study of barbiturate metabolism is the use of biologically prepared radioactive indicators in an isotope dilution procedure [Titus & Weiss (187)]. Small amounts of nine C^{14} labeled metabolites have been detected in the urine of dogs receiving pentobarbital-2-C^{14}, one of which was isolated and identified as 5-ethyl-5-(1-methyl-3 carboxypropyl) barbituric acid. Furthermore, of the two known diastereoisomers of the 3-hydroxy derivative of pentobarbital, the dextrorotatory forms the glucuronic acid conjugate to a greater extent.

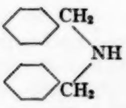
N-methyl barbiturates.—As a result of the comprehensive researches of Butler and his colleagues, demethylation in the body of *N*-methylated

hydantoins and barbiturates has been established as a general step in their metabolism. In addition Bulter raises the question of whether or not the demethylated products are the effective agents in the case of the anti-epileptic drugs, trimethadione [Butler, Mahaffee & Mahaffee (55); Butler & Mahaffee (52)], masantoin [Butler (45); Bulter & Mahaffee (53)], mephobarbital [Butler (46)], metharbital [Butler (47)], and paramethadione (50). In the case of trimethadione, quantitative studies were completed indicating accumulation with time of the demethylated product [Butler & Mahaffee (52)]. Other instances of metabolic demethylation have been described, e.g., hexobarbital (Evipal) [Bush, Butler & Dickinson (43)] and N,N[1]-dimethyl barbital [Butler (48)]. Butler's critical evaluation of the present pharmacologic and clinical status of these drugs and their demethylation products justifies a thorough reappraisal of some currently accepted notions in the use of these drugs.

Evidence that the liver is the site of demethylation is provided by the decreased rate of this process in partially-hepatectomized animals [Butler, Mahaffee & Mahaffee (54); Butler & Waddell (56)]. A finding of biochemical interest was the isolation and the identification (based on synthesis) of the metabolic oxidation product of phenobarbital (*p*-hydroxyphenobarbital) which was excreted as an acid-labile conjugate [Butler (49, 51)].

Dibenamide.—Axelrod, Aronow & Brodie (6) demonstrated that this adrenergic blocking drug (N,N-dibenzyl-β-chloroethylamine) is stored in the body fat which accounts for its long duration of action. Their other data indicate that dibenamide (IIa) probably does not undergo conversion to dibenamide alcohol but is dealkylated to dibenzylamine (IIb) which is excreted in a conjugated form, presumably by acetylation (IIc).


IIa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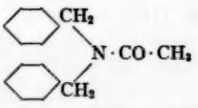
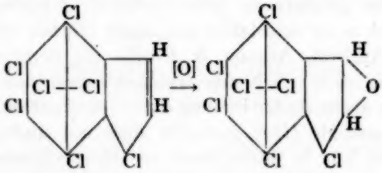

IIc

Morphine and its analogues.—Demethylation of morphine and glucosiduronic acid formation are two metabolic processes receiving study. Thus, surviving rat liver slices demethylate codeine to morphine which is then conjugated, a finding which complicates the interpretation of the action of administered codeine [Adler & Shaw (1)]. Small amounts of codeine were identified in urine of humans receiving morphine [Mannering *et al.* (117)]. Zauder (204) found that livers of morphine-tolerant rats conjugated the drug more efficiently *in vitro* as compared with control animals, but surprisingly such animals in other experiments excreted less conjugate in the urine. An extra-hepatic mechanism for drug destruction was suggested. Woods (199) found high biliary excretion of morphine (both free and conjugated) in dogs and rats, identified a phenolic glucosiduronic acid, and detected another glucosiduronic acid, uncharacterized as yet.

According to Shore and co-workers (165) about 60 per cent of *l*-2-hydroxy-N-methylmorphinan (*l*-Dromoran) administered to dogs is excreted in conjugated form in the urine, presumably as the glucosiduronic acid [Fisher & Long (85)]. Neither 1- nor 6-Dromoran are demethylated *in vivo* or by a liver demethylating enzyme system. Siebert & Huggins (167) reported that liver slices accomplished conjugation of N-allylnormorphine.

Cinchona alkaloids. — Brodie, Baer & Craig (34) isolated and identified metabolic products of the four common cinchona alkaloids, namely, 2-hydroxycinchonine, 2-hydroxycinchonidine, 2-hydroxyquinidine, and 2-hydroxyquinine. Cinchonine and cinchonidine undergo a further oxidation, the second hydroxyl being introduced into the quinuclidine ring. However, in the case of quinine and quinidine a nonphenolic monohydroxy metabolite, not the carbostyril, was the chief product and an unidentified nonphenolic dihydroxy derivative is apparently its further oxidation product.

Salicylates.—The use of C^{14} labeled salicylates is increasing in metabolic studies of the fate of this class of drugs. Alpen *et al.* (2) studied the metabolism of C^{14} carboxyl-labeled salicylic acid in dog and man, and Schayer did

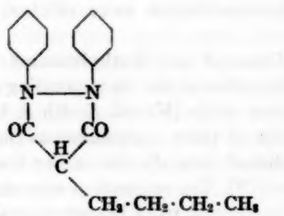


IIIa IIIb

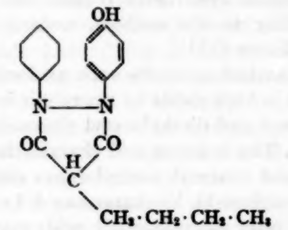
likewise (162) in the rat. Arranged in order of amount excreted, metabolites were isolated as follows: unchanged salicylic acid, glucuronic acid conjugates, salicyluric acid (*o*-hydroxy hippuric acid), and gentisic acid. Human urine contained two glucosiduronic acids, one of which retained a free salicyl hydroxyl group. C^{14}-aspirin administration led to increased blood levels of aspirin and salicylic acid in humans [Mandel, Cambosos & Smith (115)]. Of potential importance are the paper chromatographic methods developed by Quilley & Smith (144) for the study of salicylate metabolism in the rat. With regard to N-salicylates, C^{14}-salicylamide [Mandel, Rodwell & Smith (116)] is excreted by human cancer patients as its glucosiduronic acid, detection of this metabolite being made with the use of β-glucuronidase and paper chromatography [see also Becher *et al.* (14)]. The metabolism of N-acetylsalicylamide was investigated in man with respect to its urinary conjugates by Rayet and co-workers (146).

Heptachlor.—A new insecticide and a component of chlordane (IIIa) is stored in the body fat as a metabolically altered derivative which was identified as the epoxide (IIIb) [Davidow & Radomski (63)] (epoxidation, *vide infra*). Heptachlor epoxide is considered to be the intermediate which, because of its stability and lipide solubility, concentrates in the fat and may proceed later to the diol and phenol.

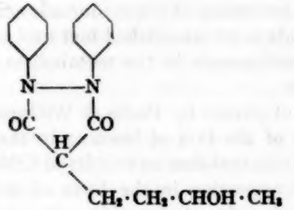
***Phenylbutazone* (*Butazolidin*).—Investigation of the physiological disposition of the antiarthritic drug, phenylbutazone (IVa), in man [Burns *et al.* (42)] was followed by the isolation and identification of two metabolites (IVb, IVc), one of them (IVc) providing another example of penultimate oxidation.**



IVa



IVb



IVc

***Other drugs.*—Space permits a listing of a few of many other drugs whose metabolism is under careful study: theophylline [Brodie, Axelrod & Reichenthal (33)], serotonin [Udenfriend & Titus (192)], arsenosobenzenes [Eagle & Doak (80)], hepatotoxic agents [Drill (76)], histamine and related imidazoles [Barnes & Denz (13)], pamaquine [Josephson *et al.* (103)] meperidine (Demerol) [Plotnikoff, Elliott & Way (143)], chloromycetin [Glazko, Dill & Wolf (95)].**

Metabolism of Some Organic Chemicals

***Aliphatic alcohols.*—A comparative study of urinary excretion products of 30 alcohols ingested by rabbits was reported by Kamil, Smith & Williams in 1953 (105). These were all glucosiduronic acids characterized by their triacetyl methyl esters. In the series of normal primary alcohols ranging from methyl- to decyl-, the amount recovered in the urine was 1 to 10 per cent of the dose, for secondary alcohols (isopropanol to secondary heptyl alcohol), the figure was 10 to 70 per cent and the tertiary alcohols (butyl, amyl, and hexyl) yielded 24, 57, and 57 per cent respectively as conjugate. No ethereal sulfates were found. Of particular interest in the metab-**

olism of methyl and ethyl alcohols was the finding of the corresponding alcohol glucosiduronic acid, isolated as the triacetyl methyl ester (1.0 per cent of dose) [Kamil, Smith & Williams (107)].

Chloroalcohols were conjugated to a greater extent with glucuronic acid as compared with the corresponding nonhalogen alcohol. The introduction of chlorine apparently renders the molecule more resistant to oxidation, according to the authors, making glucuronidation more efficient [Smith & Williams (171)].

Branched alcohols, such as 2-ethylhexanol and 2-ethylbutanol, are excreted in high yields by the rabbit in the urine as the corresponding α-ethylhexanoyl and diethylacetyl glucosiduronic acids [Kamil, Smith & Williams (106)]. The isolation and characterization of these compounds as potassium salts and triacetyl methyl esters established securely the earlier findings of Dziewiatkowski, Venkatarman & Lewis (79). The suggestion was made that stable ester glucosiduronic acids may be formed from branched-chain fatty acids when β-oxidation is blocked. This would be expected with ethyl- but not with methyl-substituted alcohols since the latter are readily oxidized to acids and beyond. The formation of glucosiduronic acids of aliphatic alcohols and branched fatty acids is an established fact and provokes speculation as to the role of glucuronidogenesis in the metabolism of naturally-occurring alcohols and fatty acids.

Benzene.—A series of papers by Parke & Williams (137 to 141) has advanced our knowledge of the fate of benzene in the organism. Altogether 84 to 89 per cent of a single oral dose to rabbits of C^{14}-benzene was accounted for, some 32 per cent appearing in the form of metabolites in the urine (140, 141). Quantitatively, phenol glucosiduronic acid was the major component of these products, polyphenolsulfates, the minor component. Attention was given this difference in type of conjugation since it was earlier suggested that the formation of polyphenols *in vivo* may be connected in some way with sulfur metabolism (137). A small amount of phenylmercapturic acid was isolated also (138).

The origin of *trans-trans* muconic acid which appears in rabbit urine following benzene ingestion was given intensive study (139). This substance was isolated and characterized as the benzhydryl ester, and specific methods for the determination of the three geometrical isomers of muconic acid were described. Since no muconic acid could be found in the urine of an animal dosed with C^{14}-phenol, it was suggested that a reduced benzene derivative, e.g., 1,2-dihydrobenzene-1,2-diol may be the precursor of *trans-trans* muconic acid. The tracking down of the metabolic fate of benzene is an important contribution to our understanding of the metabolism of aromatic rings in general, and of highly important polycyclic compounds, e.g., carcinogens, in particular.

Halogenobenzenes.—The establishment of facts regarding the fate of halogenobenzenes in the body and the development of analytical techniques for their determination is of present concern. Although, historically, the

formation of mercapturic acids has long been associated with the metabolism of halogenobenzenes, emphasis is now placed on their conversion to conjugated phenols. A systematic metabolic comparison with benzene of four different halogenobenzenes by Azouz, Parke & Williams (8) indicated that fluorobenzene in contrast to chloro-, bromo-, and iodobenzene, resembled benzene closely in the extent and type of conjugation. Surprisingly, the major metabolites of the halogenobenzenes were glucosiduronic acids and sulfates rather than the mercapturic acids. Later (9) these authors reported that between 20 and 30 per cent of monohalogenobenzenes are converted to the corresponding conjugated 4-halogenocatechols which were determined colorimetrically as blue-colored cobalt complexes. The question of perhydroxylation (*vide infra*) versus free radical hydroxylation was discussed in connection with the mechanism of this oxygenation process.

Studies have also been initiated on the metabolism of dichlorobenzene. Rabbits given *o*- and *p*-dichlorobenzene excreted a variety of phenols and catechols as glucosiduronic acids and sulfate esters [Azouz, Parke & Williams (10); Parke & Williams (142)]. With regard to *m*-dichlorobenzene, the rabbit does form a small amount of 2,4-dichlorophenylmercapturic acid and greater quantities of 2,4-dichlorophenol conjugates. The six isomeric S-dichlorophenyl-L-cysteines and the corresponding L-dichlorophenyl mercapturic acids were synthesized and certain of their physical constants recorded by Parke (136).

The use of isotopically-labeled iodobenzene in metabolic studies was reported by Mills & Wood (127). The urine of rats receiving iodobenzene-I^{131} contained two glucosiduronic acids, two ethereal sulfates, and *p*-iodophenyl-mercapturic acid, the proportion of which was unchanged by a fiftyfold variation in the dose. The presence of the glucosiduronic acid of dihydrodihydroxyiodobenzene was suspected.

Alkylbenzenes.—Penultimate oxidation has become a subject of recent interest in connection with the metabolism of the alkyl side chains of barbiturates. Both Neubauer in 1901 (133) and Thierfelder & Daiber in 1923 (186) had observed penultimate ($\omega-1$) oxidation rather than ω oxidation in the case of the conversion by rabbits of ethylbenzene to phenylmethylcarbinol.

The systematic study of the metabolism of alkylbenzenes was undertaken by Smith, Smithies & Williams (173, 174, 176) as a more general approach to the understanding of penultimate oxidation. Evidence for this route of oxidation was found for ethyl-, propyl-, butyl-, sec. butyl- and secondary pentyl-benzenes. Conjugation of the resulting alcohols with glucuronic acid was efficient. Ethylbenzene was hydroxylated in rabbits to both isomers of methylphenylcarbinol since glucosiduronic acids of both (+) and (−) methylphenylcarbinols could be isolated from the urine as triacetylmethyl esters. The lack of sulfate conjugation was attributed to the presence of only minute amounts of phenols. Furthermore, ketones derived from alkylbenzenes were reduced in the rabbit to secondary carbinols,

usually to the (−) isomer, and were excreted in conjugation with glucuronic acid.

Oxidation at both ω and (ω-1) carbon atoms was described by Robinson, Smith & Williams (154). Thus, cumene, which has narcotic but nontoxic effects was converted in the rabbit into two alcohols and one acid, all appearing as glucosiduronic acids in good yield in the urine.

Tertiary butylbenzene is metabolized almost completely to the glucosiduronic acid of 2,2-dimethyl-2-phenylethanol.

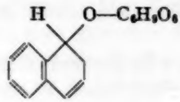
Napththalene.—Progress continues at a steady pace on the metabolic fate of naphthalene, a substance which provided one of the first examples of perhydroxylation. Thus, Corner, Billett & Young (60) found that acid treatment of a "glucuronide" fraction obtained from urine of rabbits treated with naphthalene yielded 1-naphthylglucosiduronic acid. However, incubation of this same fraction with β-glucuronidase released dextrorotatory 1,2-dihydronaphthalene-1,2-diol. Its glucosiduronic acid was subsequently isolated from the unhydrolyzed material and identified as the tetracetyl methyl ester. The ease of conversion of the dihydrodiol substance to 1-naphthylglucosiduronic acid by acid was given as the reason for the occurrence of the latter compound as the major metabolite of naphthalene, suggesting also that, in general, dihydrodiols may be immediate precursors of phenols.

Corner & Young (61) completed a study of the metabolism of naphthalene in animals of different species. The following compounds were found in the urine: free 1,2-dihydronaphthalene-1,2-diol; 1,2-dihydro-2-hydroxy 1-naphthylglucosiduronic acid; 1-naphthyl sulfuric acid; 1-naphthylglucosiduronic acid; and 2-naphthol. No major species differences were noted except in the optical activity of 1,2-dihydronaphthalene-1,2-diol present in the urine.

Using rabbits and rats, Boyland & Wiltshire (24, 25) investigated the dynamics of metabolism of naphthalene, 1-naphthol, and dihydroxydihydronapthalene. Evidence was reported that the dihydrodiol was a possible precursor of naphthol while it was unlikely that naphthol was converted to the dihydrodiol. The dihydrodiol was considered to undergo ring fission. It was further reported that liver slices slowly convert naphthalene to 1,2 dihydroxy-1,2 dihydronaphthalene but not to 1-naphthol, the latter finding being unexpected.

A careful study of dihydrodihydroxynaphthalene metabolism in the rat by Corner & Young (62) has shown that the urinary metabolites are the same as in the case of naphthalene. In addition an acid-labile precursor of 1,2-dihydroxynaphthalene was found. This metabolite was also detected in urines of naphthalene-fed rats injected with S^{35}-labeled sodium sulfate.

Recently, Boyland & Solomon (23) isolated and identified from urine of rats and rabbits given naphthalene a new class of metabolite, i.e., 1,2-dihydro-1-naphthylglucosiduronic acid (V) which is considered to originate from a route different from the one giving the dihydrodiol, since no free dihydronaphthol is excreted in contrast to free dihydrodiol.



V

Naphthylamine.—The carcinogenic properties in humans and animals of naphthylamine, an industrial chemical, has stimulated study of its metabolic fate. It is now possible, according to Henson *et al.* (100), to trace this compound in the body. Thus, rats injected with 2-(8-C^{14})-naphthylamine expired no radioactive CO_2, indicating absence of ring rupture. About 30 to 35 per cent of the administered material or its metabolites were reabsorbed from the gut. It is remarkable that after 9 to 10 weeks radioactivity was still detected in the blood cells, kidneys, and bladder.

The ability of rat liver slices to metabolize 2-naphthylamine to sulfate esters of three phenolic metabolites was studied by Booth, Boyland & Manson (21). Liver tissue possesses a mechanism for both acetylation and deacetylation but not for hydrolysis of sulfate esters.

Hydroxyquinolines and hydroxypyridines.—Thorough investigation of the metabolism of these compounds has thrown new light on metabolic hydroxylations and is of interest in connection with the metabolism and action of quinoline and antimalarials. Smith (169) isolated from rabbit urine and characterized the glucosiduronic acids of 2-hydroxypyridine and 3-, 5-, 6-, 7-, and 8-hydroxyquinolines. Quinolonylglucosiduronic acids were formed after 2- and 4-quinolones were administered.

Studies by Smith & Williams (175) provided information concerning the orientation of metabolic hydroxylations. Hydroxylation in the position *para* to the heterocyclic N atom occurred with quinoline and 2- and 4-quinolone, the products being excreted as glucosiduronic acids. However, a minor amount of 2-quinolonyl-3-glucosiduronic acid was recovered from urine of rabbits given 4-quinolone. In general, the positions of metabolic hydroxylation were similar to those found in nitrations. The authors also pointed out that in many quinoline antimalarials, the six position is blocked, leading to a reduction in antimalarial activity as compared to quinine.

The main route of quinoline metabolism in the dog appears to be to the conjugates of 3-hydroxyquinoline on the basis of studies on urine [Novak & Brodie (134)]. Recently Smith & Williams (172) have shown that rabbits excrete 3-hydroxy- and 2,6- and 5,6-dihydroxyquinolines as ethereal sulfates and glucosiduronic acids.

Aromatic nitro chemicals.—The recognition of naturally occurring nitro substituted benzenoid substances such as chloramphenicol and plant glycosides (piptagin and karakim) as well as the agriculturally useful insecticide DNOC (4,6-dinitro-*o*-cresol) has stimulated research on simpler nitro aromatic compounds, from the viewpoint [Robinson, Smith & Williams (149, 150)] that toxicity and perhaps the physiological effects of nitro com-

pounds were related to modifications which the nitro group may undergo *in vivo.*

It was first established that 70 per cent of the nitrobenzene administered to rabbits was converted to nitrophenols which were excreted as glucosiduronic acids, and 20 per cent to *o*-, *m*-, and *p*-aminophenols. These aminophenols were determined by new methods.

Anisoles.—Bray, Craddock & Thorpe (27) found that the rabbit demethylated *m*- and *p*-nitro-, *p*-chloro-, *p*-methoxy-, and *p*-cyano-anisoles to the corresponding phenols which were excreted as sulfuric and glucuronic acid conjugates in the urine. In the case of *p*-methylanisole, however, a greater proportion was converted to conjugates of anisic acid. Demethylation was demonstrated *in vitro* with rabbit liver slices.

Sulfur-containing organic chemicals.—Examples of the direction of biochemical research with respect to these compounds are given by dimethylthetin ($Me_2 \cdot S \cdot {}^{+}CH_2 \cdot COOH \cdot Cl^{-}$) and β-mercaptoethylamine. Thus, Maw (120) observed that 57 to 69 per cent of dimethylthetin chloride administered orally was oxidized to inorganic sulfate by rats and that in a series of eight such compounds, oxidation to sulfate was confined to sulfonium salts which acted as biological methyl donors. Maw therefore suggested that demethylation is an initial and obligatory step in their catabolism to sulfate. Verly and his colleagues (194) investigated the fate of S^{35}-labeled β-mercapto ethylamine (cysteamine), which is a constituent of coenzyme-A and affords good protection against irradiation of x-rays. In the dog 16 per cent of the S^{35} was excreted in the urine as sulfate, cysteamine-cystamine, and taurine [Verly & Koch (195)].

Metabolic methylation of phenols.—During a study of metabolite antagonists of thyroxine, Maclagan & Wilkinson (114) unexpectedly found a methylation of phenolic compounds. From the urine of humans but not of rabbits or rats given butyl-4-hydroxy-3,5-diiodobenzoate, the methyl ether of 4-hydroxy 3,5 diiodobenzoic acid was isolated and identified. The excretion of the methoxy acid appeared to be marked in thyrotoxicosis. This metabolic ether formation of a phenolic amino acid is an intriguing subject for future study.

Comparative metabolism of organic chemicals.—Recently, an encouraging beginning has been made in the study of the metabolism of phenols and aromatic acids by insects. This study may also be of practical importance in developing effective insecticidal phenols. Myers & Smith (132) observed first that phenol was conjugated by locusts with glucose rather than with glucuronic acid. The same is true for silkworms [Kikkawa (108)] and aphids [Brown *et al.* (40)]. On the other hand, glycine conjugates of benzoic, *p*-nitrobenzoic and *p*-aminobenzoic acids are readily formed in locusts as in the higher animals [Friedler & Smith (93)]. Smith (170) further studied the metabolism of nine phenols in locusts and detected phenylsulfuric acid in addition to phenylglucoside. Two hypotheses for explaining phenylglucoside formation have been considered [Smith (170)]. One is that locusts developed

this metabolic reaction to nullify the high β-glucuronidase activity of crop fluid and the other suggests that glucoside formation has permitted the insect to develop a glucuronidase for digesting grass hemicelluloses in the diet. Is glucosidation a step lower in biochemical evolution than glucuronidation? Such aspects of comparative metabolism of organic chemicals are thought-provoking.

Enzymes in Metabolism of Drugs and Organic Chemicals

One important aim in all of biochemistry is to elucidate at the cellular level the enzymic mechanism responsible for a given metabolic alteration in the chemical constitution of a compound. In the case of foreign organic chemicals, in general, these systems are of interest to the extent that they explain the transformations of the molecule aud throw light on the biochemical potentialities of the cells which may not have been recognized ordinarily. For drugs there is the additional desideratum, to explain their mode of action enzymatically. Also, many drugs and organic chemicals have important effects on enzyme systems not directly related either to their metabolism or to pharmacologic action. Discussion will be limited to examples of the first two aspects, dealing in order with mode of action of drugs, dealkylation, deamination, mercapturic acid synthesis, metabolic conjugation with sulfuric and glucuronic acids, sulfatase, and β-glucuronidase.

Mode of action.—Some idea of the detail with which it may be possible to delineate the cellular sites of action of drugs in biochemical terms is demonstrated by Zatman and co-workers (202, 203). Isonicotinic acid hydrazide (INH), the anti-tuberculosis drug, is a potent inhibitor of beef spleen diphosphopyridine nucleotidase. That this phenomenon is specific for INH is supported by the fact that both nicotinic acid hydrazide and nicotinamide are much less inhibitory. In the enzyme digests an INH analogue of DPN has been synthesized from the reaction of DPN and INH, and it was later isolated in 75 per cent yield. The authors state that "as far as explaining the the antituberculosis action of INH is concerned, analogue formation by the host tissues, if not by the bacterium itself, might conceivably provide an answer." In searching for explanations of the site and mode of action of certain drugs, it may be profitable, as in this case, to look for chemical combinations between drugs and coenzymes.

Other workers have studied in lesser detail the effect of barbiturates on oxidative phosphorylation [Brody & Bain (39)] on adenosinetriphosphatase [Maxwell & Nickel (121)], of light-activated benzyprene on urease [Mills & Wood (126)], and of diethyl *p*-nitrophenyl phosphate on cholinesterase [Gage (94)].

Enzymatic dealkylation.—Considerable progress has been made in outlining the enzyme systems of liver which accomplish dealkylation of N-alkyl drugs. La Du *et al.* (109) reported that aminopyrine and its ethyl and butyl homologues are dealkylated in rabbit, rat, and guinea pig liver homogenates to yield 4-aminopyrine. The methyl groups are oxidized to

formaldehyde and the ethyl group to acetaldehyde. Both TPNH and oxygen are required, and the dealkylation system is located in the microsomes. The rat liver has a similar system for demethylating 4-dimethylaminoazobenzene but requires both DPN and TPN [Mueller & Miller (131)].

Enzymatic deamination.—The microsomal fraction of rabbit liver in the presence of reduced TPN and oxygen deaminates amphetamine to phenylacetone and ammonia [Axelrod (5)]. This enzyme system is stated to differ from other liver deaminases.

Other observations.—β-Diethylaminoethyl diphenylpropylacetate hydrochloride ("β-di") prolonged the action of a variety of drugs by inhibiting their metabolism [Axelrod, Reichenthal & Brodie (7)]. Cooper, Axelrod & Brodie (59) state that "β-di" inhibits the enzyme systems in liver slices and homogenates which oxidize the side chains of barbiturates; dealkylate merperidine, dibenzamine, and aminopyrine; deaminate amphetamine; cleave the ether linkage in codeine; and conjugate phenol and morphine. The possible importance in therapeutics of nontoxic drug metabolic inhibitors such as "β-di" makes it necessary to examine critically the experimental findings. The data would have been more convincing, at least to this writer, if in addition to drug disappearance, the authors had measured one or more products of any one of the five reactions they studied. Moreover, "β-di" has no effect on hydrogen transport and on known TPN-requiring enzymes in homogeneous solution. It would seem necessary to prepare purified individual enzymes from the microsomes and then demonstrate the inhibitory action of "β-di" before one could ascribe to it specificity of a true enzyme inhibitor. The possibility that "β-di" may have relatively nonspecific physical effects on microsomes suggests an alternative unexplored explanation.

Mercapturic acid synthesis.—Mercapturic acid synthesis *in vitro* has now been described by Mills & Wood (128). In the presence of iodobenzene I^{131} dispersed in rat plasma, rat liver, and kidney slices synthesized *p*-iodophenylmercapturic acid. This was crystallized to constant specific activity following the addition of nonlabeled carrier. However, quantitatively, hydroxylation was more extensive than mercapturic acid formation, irrespective of dosage. These findings should provide an approach to the study of the enzymatic mechanism of mercapturic acid formation.

Sulfate ester formation.—From the work of Bernstein & McGilvery (15, 16), Segal (164), and De Meio *et al.* (66, 67), the following enzymatic mechanism for sulfate ester formation seems probable.

$$SO_4^{=} + \text{ATP} \rightarrow \text{active sulfate}$$

$$\text{Phenol} + \text{active } SO_4 \rightarrow \text{phenol sulfate}$$

That inorganic sulfate may be utilized by the whole animal for sulfate ester synthesis was proved by Laidlaw & Young (110) who isolated radio-

active 2-amino-1-naphthylsulfuric acid and 2-naphthylsulfuric acid from the urine of rats given the phenols and S^{35}-labeled sodium sulfate.

A consideration of some interest is whether or not sulfate ester and glucosiduronic acid synthesis of phenols and alcohols bear an inverse relationship to each other. Storey's data (177) on *o*-aminophenol conjugation by liver slices and De Meio & Tkacz (65) on phenol conjugation favor this proposition, wherea Sie & Fishman's work (166) with *m*-aminophenol does not. Dodgson, Rose & Spencer (71) have employed 5-aminoacridine to precipitate biosynthetic arylsulfates, a convenient step in their isolation and identification.

Kinetic studies of conjugation in vivo.—Bray and his colleagues have provided data concerning rates of a variety of metabolic processes which benzoic acid, its precursors and phenols and other compounds undergo in the rabbit. The development of mathematical expressions for treating these data is of undoubted importance [Bray, Thorpe & White (31, 32); Bray *et al.* (28, 29, 30); Bray (26)].

Enzymatic Synthesis of Glucosiduronic Acids

A single sequence of reactions leading from trioside formation to glucosiduronic acid via two successive condensations of 3 carbon sugar intermediates with the aglycone has been the generally accepted mechanism of glucosiduronic acid formation [Lipschütz & Bueding (113)]. It now appears that glucosiduronic acid synthesis is a two-step process, the first involving production of glucuronic acid and the second, its utilization in the formation of the conjugate.

Glucuronic acid is synthesized in the organism directly from glucose. Evidence for this statement is based chiefly on the C^{14} tracer work of Mosbach & King (130), Eisenberg & Gurin (81), and Douglas & King (74, 75) who showed that the labeled carbons of glucose could be detected in the carbon chain of glucuronic acid in borneol and menthol glucosiduronic acids isolated from guinea pig and rabbit urine [see also Bidder (17); Packham & Bulter (135); Bulter & Packham (44); Doerschuk (73)]. It is of interest that glucose is the precursor of glucuronic acid in hyaluronate synthesized by Streptococcus [Roseman *et al.* (156, 157)].

Recent work has thrown some light on a pathway which seems to explain the route, glucose to glucuronic acid. Liver contains an enzyme system, which will oxidize glucose present in uridinediphosphate glucose (UDPG) to the glucuronic acid analogue (UDPGA) [Strominger *et al.* (179)]. Experimental evidence for the presence of UDPGA in mammalian liver has been reported recently by Dutton & Storey (78), Story & Dutton (178), Smith & Mills (168), and Hurlbert & Potter (101).

The second step, glucuronic acid to glucosiduronic acid, may proceed by two pathways. Thus, Dutton & Storey (78), Smith & Mills (168), and Isselbacher & Axelrod (102) have demonstrated glucosiduronic acid syn-

thesis by cell-free liver preparations in a system containing UDPGA and a suitable alcohol or phenol to serve as an acceptor molecule, presumably catalyzed by a transferase enzyme which does not have the properties of β-glucuronidase, according to this equation:

$$\text{UDPGA} + \text{Acceptor} \rightarrow \text{UDP} + \text{Acceptor:glucosiduronic acid}$$

A second route (glucuronic acid to glucosiduronic acid) requires the participation of β-glucuronidase:

$$RO \cdot C_6H_9O_6 + R'OH \xrightarrow{\beta\text{-glucuronidase}} ROH + R'OC_6H_9O_6$$

By means of a specific sensitive method for measuring both glucuronic and glucosiduronic acid in mixtures, β-glucuronidase has been observed by Fishman & Green (89) to catalyze efficiently the transfer of glucuronic acid from its conjugate to a suitable acceptor molecule. This type of transfer reaction has been noted before with respect to glucosidases, nucleases, phosphatases, proteolytic and other enzymes, and is considered by many to be of physiological significance.

Evidence exists for two distinct types of glucosiduronic acid synthesizing enzyme systems, and it remains to be shown to what extent each participates in glucosiduronic acid synthesis in the organism.

While the UDPGA-transferase mechanism for glucosiduronic acid synthesis represents an important advance in our knowledge of this metabolic reaction, it does not explain a number of findings. Thus, saccharate which does not interfere with this cell-free system [Dutton & Storey (78)] is an inhibitor of the synthesis by surviving liver slices of *o*- and *m*-aminophenyl glucosiduronic acid [Storey (177); Sie & Fishman (166)]. Moreover, as shown by Flock & Bollman (92), extra-hepatic synthesis of thyroxine glucosiduronic acid in the eviscerated rat is greater than that observed in the intact animal, a fact which is difficult to explain by the UDPGA-transferase mechanism located only in liver.

Arylsulfatase.—Interest in this enzyme is directed at the level of study of its properties and kinetic behavior as well as developing a specific hydrolytic agent for the ester sulfate linkage. Richest sources of the enzyme have been found in the lower forms of living organisms, e.g., molluscs, snails, insects, and bacteria. Robinson and co-workers (151, 152) reported that takadiastase arylsulfatase hydrolyzed the alkali salts of *o*-, *m*-, *p*-nitro, *p*-chloro-, *p*-aldehydo-, 4-hydroxy-2-nitro-, 4-hydroxy-3-nitro-, and 2-hydroxy-5-nitro-phenylsulfuric acids. Kinetic studies established values for Km, optimum pH, optimum substrate concentration, and rates of hydrolysis for all of these substrates. Marine molluscs are rich in both β-glucuronidase and arylsulfatase [Dodgson, Lewis & Spencer (69); Dodgson & Spencer (68)]. These authors report methods for separating the two enzyme activities so that kinetic measurements of sulfatase became possible. Locust crop liquor arylsulfatase hydrolyzed 4-nitrocatechol sulfuric acid [Robinson, Smith & Williams (153)]. A systematic survey revealed substantial arylsulfatase

activity in certain strains of *Salmonella* and *Mycobacteria* [Young, Morrison & Whitehead (201); Whitehead, Morrison & Young (196)]. A sulfatase-rich microorganism present in tidal mud is now believed to explain the presence of high sulfatase activity in molluscan digestive organs [Dodgson *et al.* (70)]. Mammalian liver arylsulfatase has also been investigated [Dodgson, Spencer & Thomas (72); Roy (158)].

β-Glucuronidase.—The rather extensive literature on β-glucuronidase has been reviewed recently [Fishman (87); Levvy (111); Bray (26); Teague (185)]. This enzyme is of interest principally because of its wide tissue distribution, its sensitivity in certain tissues to gonadal hormones, its control in organs of the mouse by genetic factors, and its occurrence in abnormal amounts in cancer tissue in the majority of instances. It has become increasingly important to define the exact function of the enzyme in tissue in order to interpret properly its place in these various biological phenomena. Of practical importance is the use of preparations of β-glucuronidase from sources as diverse as liver, snails, and bacteria as a tool for isolating, identifying, and analyzing the aglycones (e.g., drugs, steroids, phenols, etc.) of glucosiduronic acids.

A recent contribution to the study of the specificity of β-glucuronidase is the demonstration by Levvy & Worgan (112) of enzymic hydrolysis of 1-O-acyl glucosiduronic acids. Also, Dutton (77) has reported that fatty acid glucosiduronic acids appear to be synthesized by a cell-free UDPGA system.

Glucosiduronic acids *per se* need not be associated with questions of toxicity since they are normal constituents of certain plants. Recently Marsh (118) has begun a systematic investigation of plant glucosiduronic acids and their hydrolysis by β-glucuronidase. This field has been dormant since Miwa's reports in 1934 (129).

Glucuronic acid metabolism.—With the availability from commercial sources of pure glucuronic acid and its lactone much work on the metabolism of glucuronic acid in humans and animals has appeared. The time course of utilization and excretion of glucuronolactone ingested by man in health and in certain disease states has been outlined [Fishman *et al.* (91); Sudhof (180); Sudhof & Schellong (181)]. Effects of menthol administration on urinary glucuronic acid in various disease states was studied by Brox (41).

It is now possible to measure serum glucosiduronic acids [Fishman & Green (88)] without interference by nonglucuronic acid, naphthoresorcinol positive substances. Normal values vary in the neighborhood of 1 mg. per cent. The time course of conjugation with glucuronic acid of drugs and organic chemicals can be conveniently studied.

The existence of free glucuronic acid in the body is not settled. While Artz & Osman (4) in their review of the literature did not report free D-glucuronic acid in nature, Teague suggests that a considerable proportion of serum glucuronic acid is free, on the basis of low values for *n*-butyl acetate-extractable glucuronic acid (conjugated) when compared with figures vari-

ously reported for total glucuronic acid [DeFrates & Boyd (64)]. From Fishman & Green's work, it is probable that this "free" fraction is attributable to glucose and other nonglucuronic acid materials which give color interference. This would explain the high serum glucuronic acid values reported in diabetes [Saltzman, Caraway & Beck (160)]. Their search (88) for free glucuronic acid in the blood by chromatographic techniques was unsuccessful.

L-Xylulose was identified in essential pentosuria in 1945 [Enkelwitz & Lasker (83)] and shown to be, in all probability, a urinary metabolite of glucuronic acid. Recently, Touster, Hutcheson & Rice (188) found trace amounts of this pentose in guinea pig and human urine and caused an increased excretion by the administration of glucuronolactone. The fact that liver and kidney contain an enzyme system which decarboxylates glucuronolactone and glucuronic acid may be relevant [Rabinowitz (145)].

Chemistry of the glucosiduronic acids.—A service has been done by the collection and classification of all known glucosiduronic acids along with their characteristic constants [Williams (198); Bray (26); Teague (185)]. Of value also is a paper by Kamil, Smith & Williams (104) who have outlined a general method for the preparation of glucosiduronic acids and their derivatives. After initial purification of the lead glucosiduronates, the free acids are esterified and acetylated. These triacetyl methyl esters are cystalline and easily characterized. The preparation of the glucuronidamides, also described, may be preferred by those who wish to regenerate the acid without serious losses. Some physical constants of glucosiduronic acid amides and triacetyl methyl esters of a variety of cresols and of halogenated phenols are reported [Robinson, Smith & Williams (153)].

One explanation for the glucuronic acid conjugation mechanism, first proposed by Quick, holds that the conjugated material usually exhibits stronger acidity than the aglycone, thus making the conjugate easier for the body to excrete. The most complete examination of this thesis and of other similar ideas was made (153) and the apparent dissociation constants, absorption spectra, and optical rotation values for 21 biosynthetic β-glucosiduronic acids were recorded. The pK_a for these compounds were all between 3 and 4 and close to glucuronic acid (2.93). Of interest were the observations that whereas hippuric acid formation was limited to carboxylic acids with pK_a from 2 to 5, and ethereal sulfates limited to phenols whose dissociation constants ranged from 6 to 11, the formation of glucosiduronic acids took place irrespective of the value for pK_a.

The necessity of utilizing animals to synthesize glucosiduronic acids is a factor limiting the investigator who wishes to be certain of obtaining the glucuronic acid conjugate of a chosen compound. In this regard a number of reports have appeared detailing the laboratory synthesis of glucosiduronic acids which may remove this obstacle. Marsh (119), Barker, Bourne & Stacey (12), and Tsou & Seligman (190, 191) oxidized glucosides to the corresponding glucosiduronic acids using oxygen in the presence of platinum

black. Modifications of the classical procedure of Goebel & Babers (96) have been published which in the hands of Bollenback and co-workers (20) have yielded a variety of aryl glucosiduronic acids and in the laboratory of Taurog, Abraham & Chaikoff (183) the O-glucosiduronic acids of tyrosine, diiodotyrosine, and triiodothyronine, all as the triacetyl methyl esters.

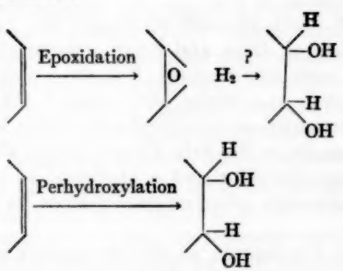
Of interest also are the syntheses of β-D-glucuronic acid-1-phosphate, reported by Touster & Reynolds (189), glucuronic acid analogues of pyrimidine nucleosides by Goodman (97), and D-glucuronolactone isonicotinyl hydrazone by Sah (159).

Comments

It appears desirable to bring into focus the implications of some recent developments which may apply not only to the metabolism of drugs and organic chemicals but also to important body constituents.

Penultimate oxidation.—To the well known ω and $\omega-2$ (β) oxidation of hydrocarbon chains there are now a number of well-documented instances of $\omega-1$ (penultimate) oxidation, e.g., barbiturates. One is reminded of the classical studies of Knoop who administered fatty acids connected to a difficultly oxidizable aromatic ring and identified the urinary metabolites. This study of the metabolism of a class of foreign organic chemicals provided the basis of our understanding of fatty acid metabolism (β-oxidation). On the basis of this historical precedent the fact of penultimate oxidation of hydrocarbon chains should direct attention to the metabolism of naturally-occurring substances which possess hydrocarbon chains, e.g., aliphatic alcohols, cholesterol, and certain antimalarial alkyl-substituted naphthoquinones.

Epoxidation and perhydroxylation.—The formation of an epoxide (VIa) is considered by Boyland to be the first step in the oxidation of aromatic rings, a vewpoint developed to explain the carcinogenic activity of polycyclic carcinogens [Boyland (22); Haddow (98)]. Apart from naturally-occurring epoxides, e.g., violaxanthin and hepaxanthin (carotene epoxide), heptachlor epoxide represents the first example of the isolation in the body of an epoxide metabolite.



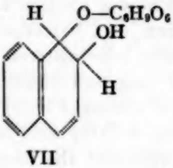
VIa

VIb

VI

The mechanism of formation of dihydrodiols (VIb) need not necessarily proceed via an epoxide intermediate but may result from free radical hydroxylation. Certain epoxides [Everett & Kon (84)] effect biological changes resembling markedly the radiomimetic mustards, and in this connection 1,2,3,4, diepoxybutane is carcinogenic. This biological reactivity of epoxides may explain the fashion in which the chemically-inert carcinogenic hydrocarbons exhibit their powerful biological activity.

Perhydroxylation and glucuronidogenesis.—From the careful study of the metabolism of aromatic hydrocarbons, a phenomenon of partial ring saturation with dihydroxysubstitution has emerged. This has been termed perhydroxylation because it involves the addition of the elements of hydrogen peroxide to the molecule. Examples of this metabolic reaction are the formation and excretion by the rabbit given anthracene or naphthalene of 1,2-dihydro-1,2-dihydroxyanthracene D-glucosiduronic acid and 1,2-dihydro-



VII

2-hydroxy-1-naphthyl D-glucosiduronic acid (VII). A property of these dihydrodiol compounds whether free or conjugated, is the ease of dehydration to phenols or phenolic glucosiduronic acids. Boyland (22) and Young (200) have reasoned, therefore, that many phenolic compounds excreted in the urine following the administration of various benzanthracenes were derived from intermediate epoxy compounds, labile diols, or both. They may be regarded as the first products of oxidation of aromatic ring compounds. Robinson, Smith & Williams favor this mechanism also for explaining the production of polyphenols arising from the metabolism of substances such as phenol and nitrobenzene.

The frequency with which glucosiduronic acids are encountered in the study of the metabolism of drugs and organic chemicals emphasizes the general nature of this metabolic reaction. The same also holds for sulfate ester formation. However, the firmly held concept that these products represented merely inactivated or detoxified metabolites has had the effect of inhibiting original inquiry in this field. Consequently, the ideas described below offer a refreshing viewpoint and a challenge to the experimenter, even though they require more experimental evidence for complete acceptance.

Thus, Teague (185) has introduced the provocative concept that conjugation with D-glucuronic acid and sulfuric acid are essential processes in the perhydroxylation of aromatic rings. Activation of the molecule during

oxidation is considered to give rise to a nascent hydroxyl group which then can be more readily conjugated. Thus, explanations are provided for several perplexing problems. Examples are the fact that bromobenzene yields more sulfate ester conjugate than does bromophenol, and that more 1-O-acyl-D-glucuronic acid arises from certain aromatic aldehydes rather than from the corresponding acids. Teague points also to the analogy in the metabolism of chloral and bromal hydrates. Thus, bromal hydrate is apparently converted directly to tribromoethyl-D-glucosiduronic acid without evidence of the formation either of tribromoethanol or tribromoacetic acid. Now, Boyland & Solomon (23) have found 1,2-dihydro-1-naphthyl glucosiduronic acid but not the free hydroxy compound after naphthalene dosage.

The biologically functional groups of drugs and organic chemicals are the sites on the molecule which not only govern lipide solubility, surface tension, and pH of its solutions, but are also the locations which undergo metabolic change. Teague defines enzyme systems of "metabolic" or "biological activity" but points out carefully that in some cases the two systems may be the same. Thus, monamine oxidase which oxidatively deaminates epinephrine may be involved in the reactions accounting for adrenergic cell stimulation. Conjugation with glucuronic or sulfuric acids is regarded as a necessary step in the metabolism of organic compounds which at some time possess a reactive hydroxyl group. The question arises as to whether or not the enzyme systems affecting glucosiduronic acid formation may, like monamine oxidase, be implicated in the biological, pharmacological, and toxic actions of the aglycones, whether of exogenous or endogenous origin.

Certainly, these ideas, together with the hypothesis of biological antagonism (*vide infra*) will do much to counteract the paralysis of original thinking in the area of biochemical significance of conjugation mechanisms.

Hormone conjugates.—An increasing number of instances have been recorded in which the organism forms conjugates of hormones and their metabolites. This is a well-recognized phenomenon for steroid hormones and their metabolites, a few of which may be mentioned: estriol glucosiduronic acid [Cohen & Marrian (58)], estrone sulfate [Schachter & Marrian (161)], the glucosiduronic acids of pregnanediol [Venning & Browne (193)], and tetrahydrocortisone [Schneider (163); Bagget, Kinsella & Doisy (11)].

Of more recent vintage are reports of glucosiduronic acids of epinephrine [Clark *et al.* (57)] and norepinephrine [Elmadjian, Lamson & Neri (82)], of thyroxine [Taurog, Briggs & Chaikoff (184)], and triiodothyronine [Roche, Michel & Tata (155)], and of testosterone [Fishman & Sie (90)]. A number of these have been detected in urine, in bile, or in tissue slice incubation media.

Two possible functions for the hormone conjugates may be considered. The conjugates may represent the form in which the hormone is utilized by the "target" tissue, or they may be transport combinations which preserve the hormone from oxidative attack by other tissues. These possibilities have been discussed by this reviewer and others [Fishman (86); Roberts & Szego

(147, 148); Szego & Roberts (182)]. Attention should also be directed to possible epoxides and other intermediates in the hydroxylation and conjugation of steroids, e.g., epoxidation of unsaturated steroids by microorganisms [Bloom & Shull (18)].

Regardless of whether these hormone conjugates are biologically active, it is important to study the mechanisms of their formation inasmuch as the extent of action of a hormone is directly related to its blood level, and this in turn may well depend on the rate of hormone conjugation.

Significance of metabolic mechanisms associated with fate of drugs and organic chemicals.—A rebirth, in a new form, has been noted of the hypothesis that the organism in its evolution has perfected specific metabolic mechanisms for the detoxication of drugs [Ambrose & Sherwin (3); Harrow & Sherwin (99)]. Brodie and his colleagues have coined the term "drug enzyme systems" in referring to liver microsome suspensions which catalyze a variety of transformations, e.g., dealkylation, hydroxylation, etc., and have speculated that these enzyme systems may not be "essential for the normal economy of the body, but counteract the toxic influences of foreign compounds that gain access to the body from the alimentary canal" [Brodie *et al.* (35)].

The reader should be reminded that the so called detoxication mechanisms have been assigned a more general biochemical significance in recent years. Much of early biochemistry dealt with the isolation and characterization of urinary excretion products following the administration of drugs and organic chemicals. Consequently, it was the custom to connect metabolic mechanisms such as conjugation and ring oxidation, with the fate of these compounds. Most instances of these same metabolic changes in normal body constituents were recorded much later, principally because these substances occur in low concentration in the blood and urine and have required more effort and refined techniques for their isolation and identification. Consequently, it does not seem necessary to posulate, for instance, that the organism has one mechanism for synthesizing the sulfate ester of a phenol of endogenous origin, e.g., estrone, and another for the sulfate ester of a phenol absorbed from the gastrointestinal tract. The same applies to all other metabolic reactions referred to in this review, even though in some cases the endogenous natural substrate has yet to be identified. It is clearly logical to assume that the enzyme machinery of the body does not distinguish between exogenous chemicals and its own constituents when these have a similar architecture. This view also is developed in detail by Teague (185) who termed it the "biological-antagonism" concept. Moreover, many biologically and pharmacologically inert compounds may share the same metabolic fate in the body as drugs and hormones, a fact which further points up the inadequacy of the detoxication viewpoint in explaining the significance of these reactions.

It may be helpful to define the different approaches to the study of drugs and organic chemicals. Thus, the classical pharmacologist's principal interest is evident. It lies in the drug, its action, its toxicity, and its fate in the body,

The biochemical approach, pursued also by modern pharmacologists, has been less clearly defined and requires elaboration. It is seen as developing along the following lines: (*a*) A study of the nature of the urinary products resulting from the administration of drugs or organic chemicals is necessary to obtain a broad base of information. (*b*) A single compound may be chosen for study because of its particular interest, e.g., a carcinogen, an antibiotic, a steroid, and here, the primary objective may be to explain the biological activity of the agent. (*c*) The purpose may be that of using an organic chemical as a means of investigating some aspect of intermediary metabolism, e.g., use of aromatic amines in the study of acetylation. (*d*) Enzymatic mechanisms are being studied either in relation to the metabolism and mode of action of drugs or to their effects on specific enzyme systems. Thus, there is a trend to fuse the subject of metabolism of drugs and organic chemicals with that of intermediary metabolism in general.

With regard to biochemistry, the present objective, somewhat unclear in the past, is now to define in precise enzymatic terms not only the reactions which drugs and organic chemicals undergo, but the reactions which are responsible for drug action. While pursuing these aims, one should not lose sight of the possibility of utilizing the findings as tools in studying intermediary metabolism.

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CARBOHYDRATE METABOLISM[1,2]

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On December 10, 1955, when Seymour Korkes lost his life in an automobile accident, this review was in varying stages of completion. In tribute to his memory a group of his friends[3] volunteered to complete the review. His notes have served as the nucleus of each section of the review and determined the areas of carbohydrate metabolism to be considered. They also provided the original ideas and viewpoints to be found in the text. The review below is presented without apology but with deep regret that the incisive thought and imagination of the original author has been lost to Biochemistry.

I. POLYSACCHARIDE SYNTHESIS

Mediation by uridine nucleotides.—The participation of uridine nucleotides in glycoside synthesis has been substantiated in a number of laboratories. Leloir & Cardini have observed two separate sucrose synthesizing systems in wheat germ (1, 2) which catalyze the reactions:

$$\text{UDPG}^{2} + \text{fructose} \rightarrow \text{sucrose} + \text{UDP} \qquad (a)$$

$$\text{UDPG} + \text{fructose-6-phosphate} \rightarrow \text{sucrose phosphate} + \text{UDP} \qquad (b)$$

The phosphate of the sucrose phosphate appears to be esterified in the C_6 position of the fructose moiety. The operation of (*b*) rather than of (*a*) or of the sucrose phosphorylase of *Pseudomonas saccharophila* (3) would explain the observation that sucrose synthesized from glucose-1-C^{14} by wheat seedlings was equally labeled in both the glucose and the fructose moieties, while the free fructose in the cells did not become significantly labeled (4). The data presented by Turner (5) do not permit a decision as to which of the

[1] The survey of the literature pertaining to this review was completed in December, 1955.

[2] The following abbreviations are used in this chapter: ADP for adenosinediphosphate; AMP for adenosinemonophosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; DPNH diphosphopyridine nucleotide (reduced form); EM for Embden-Meyerhof; FAD for flavin-adenine-dinucleotide; FDP for fructose diphosphate; F-6-P for fructose-6-phosphate; G-1-P for glucose-1-phosphate; G-6-P for glucose-6-phosphate; HMP for hexose monophosphate; IDP for inosine diphosphate; ITP for inosine triphosphate; P_i for inorganic orthophosphate; P-P_i for inorganic pyrophosphate; PGA for 3-phosphoglyceric acid; RuDP for ribulose diphosphate; R-5-P for ribose-5-phosphate; S-7-P for sedoheptulose-7-phosphate; TCA for tricarboxylic acid; TPN for triphosphopyridine nucleotide; TPNH for triphosphopyridine nucleotide (reduced form); UDP for uridine diphosphate; UDPG for uridine diphosphoglucose; UDPGal for uridine diphosphogalactose; UMP for uridylic acid; UTP for uridine triphosphate.

[3] W. L. Byrne, S. P. Colowick, P. Handler, B. L. Horecker, N. O. Kaplan, S. Kaufman, F. Neuhaus, E. Racker, and G. W. Schwert, Jr.

two Leloir & Cardini systems was responsible for sucrose synthesis in a glycolyzing pea extract which accumulated sucrose when incubated with glucose-1-phosphate and fructose, provided that DPN, UMP, and ATP were present, but it is apparent that the *Pseudomonas* type sucrose phosphorylase was not the responsible agent.

Glaser & Brown (6) have reported the synthesis of labeled oligosaccharide chains composed of N-acetyl-D-glucosamine and D-glucuronic acid units, combined as in hyaluronic acid, upon incubation of homogenates of Rous chicken sarcoma with UDPG and either N-acetyl-C^{14}-D-glucosamine plus UTP or UDP-N-acetyl-C^{14}-D-glucosamine. The source of glucuronic acid units was presumed to be UDP-D-glucuronic acid formed by the DPN-dependent (7) oxidation of the UDPG present in the incubation mixtures. The resultant polysaccharide was degraded with testes hyaluronidase to N-acetyl-C^{14}-hyalobiuronic acid and the corresponding C^{14}-tetrasaccharide. The enzymes responsible for this polysaccharide synthesis were not isolated; however, the preparations were shown to contain the systems required for synthesis of the supposed immediate reactants from UDPG and glucosamine-6-phosphate. The total synthesis observed was of a low order of magnitude, and it is unfortunate that the C^{14}-label was in the N-acetyl group so that the possibility of acetyl exchange from the added substrates to pre-existing hyaluronic acid or its precursors could not be precluded, particularly since after administration of acetate-1-C^{14} to rabbits, the radioactivity of the hyaluronic and chondroitin sulfuric acids of skin was confined almost entirely to the N-acetyl moiety (8) indicating much more extensive turnover of the N-acetyl group than of the sugar fragments. However, the results reported are highly suggestive, and a more definitive report is awaited with much interest.

A similar mechanism may be operative in the synthesis of 4-0-β-D-galactopyranosyl-N-acetyl-D-glucosamine from lactose and N-acetyl-D-glucosamine by *Lactobacillus bifidus* (9) although the data are compatible with simple transgalactosidation. The required UDPGal may be presumed to arise thus: UTP+Gal-1-P→UDPGal+P-Pi as shown to occur in *Saccharomyces fragilis* (10). Additional evidence for the substrate specificity of the enzymes involved is found in the observation that extracts of *Hemophilus influenzae* cleave UDPG to UMP, uridine, and G-1-P whereas UDP-N-acetyl-glucosamine is unaffected (11).

Dutton & Storey (12) have isolated the factor which they had previously found to be necessary for hepatic glucuronide synthesis and identified it as UDP-glucuronic acid. Smith & Mills (13) independently arrived at the same conclusion. This reaction, therefore, is depicted as: UDP-glucuronic acid+ROH→glucuronyl-OR+UDP. The former authors speculated that, if UDP-glucuronic acid is also involved in mucopolysaccharide synthesis, the formation of glucuronides of the numerous compounds with no structural similarity but an hydroxyl group may be entirely "accidental." In any case, the concatenation of various observations to date warrants intensive investiga-

tion of the likelihood that uridine derivatives are the immediate substrates for these and other instances of polysaccharide formation.

Knowledge of the existence and metabolic interrelations among the various uridine derivatives required in these transformations have been extended in the period under review. The system for oxidation of UDPG to UDP-glucuronic acid has been isolated (7) and found to entail DPN reduction. Curiously, there was no indication that this oxidation of a primary alcohol to a carboxylic acid requires more than one enzyme nor was there any indication of the transitory aldehyde which might be expected. Attempts to demonstrate the formation of UDP-glucuronic acid by other pathways such as UDPG or UTP reacting with glucuronic acid have, thus far, failed. Glucuronolactone also appears to be of no metabolic significance in this regard (14, 14a). Further work on glucosone as an intermediate in the conversion of glucose to glucosamine (15) has been interpreted in a manner contrary to previous suggestions (16). A significant fraction of glucosone-C^{14} (C_1 or uniformly labeled) added to growing cultures of a strain of group A hemolytic streptococci was converted to compounds other than glucosamine, and the glucosamine isolated from hyaluronic acid had 1/3 to 1/5 of the expected radioactivity. Thus, these studies do not support the role of glucosone as an intermediate in glucosamine synthesis. On the other hand, evidence has continued to accumulate that glutamine is the nitrogen donor for the precursors of hyaluronic and chondroitin sulfuric acid biosynthesis (17, 18, 19). There is no doubt that glucosamine may be activated and incorporated into hyaluronic acid, however. Indeed, the nitrogen of glucosamine does not appear to be in equilibrium with the general cell nitrogen of hemolytic streptococci which retain the isotope when incorporating glucosamine-N^{15} into hyaluronic acid (20).

UDP-N-acetylglucosamine, thought to be present in liver (13, 21) and previously found in yeast (22), has been isolated from extracts of bovine liver (23). UDP-N-acetylgalactosamine was also isolated from these extracts (23) while UDP-N-acetylgalactosamine sulfate, necessary for chondroitin sulfate synthesis, has been obtained from oviduct, together with UDP-N-acetylglucosamine-6-phosphate (24) whose function is presently unknown.

Park (25) in 1952 described three uridinediphosphate derivatives which had in common an unidentified component and noted that an unknown compound with similar properties was present in certain hyaluronic acid preparations. Based on current information, the unidentified component of the Park nucleotides is very similar to sialic and neuraminic acids which, with related compounds, have been found in appreciable quantities in a great variety of sources (26, 26a, 26b). It seems likely that one function of the Park nucleotides is to serve as the activated form of yet another component of mucopolysaccharides, sialic acid. Gottschalk (27) has summarized the available chemical information and has proposed structures for sialic acid (see Fig. 1) and neuraminic acid. The wide distribution and unusual properties of

these compounds indicate that a new and interesting chapter in metabolism will shortly be written.

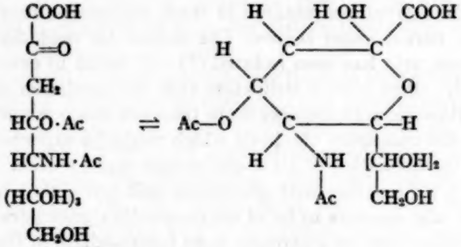


FIG. 1. Sialic acid.

Glycogen phosphorylase.—The interconversion of phosphorylases *a* and *b* has been subjected to careful scrutiny. Partial purification of the phosphorylase-rupturing enzyme (PR) from rabbit muscle has been reported by Keller & Cori (28). Noteworthy among its properties is the fact that phosphorylase *a* is protected by the presence of its usual substrates, G-1-P and priming oligosaccharides. The PR enzyme exhibited no hydrolytic action on crystalline myosin or aldolase nor on several typical synthetic substrates for trypsin (29). However, among the latter, the ethyl esters of arginine and lysine proved to be competitive inhibitors for the conversion of phosphorylase *a* to *b*. The same laboratory reported (30) that samples of phosphorylase *b*, prepared from phosphorylase *a* by the action of either PR enzyme or trypsin, showed only minor differences, the former having a molecular weight of 257,000 and the latter 242,000 as determined by sedimentation. Electrophoretically, however, the two phosphorylases were distinct, that resulting from tryptic activity migrating to the anode at pH 7.8 considerably more rapidly than did phosphorylase *b* resulting from PR enzyme activity.

Sutherland & Wosilait (31) have reported partial purification of both a glycogen phosphorylase and a phosphorylase-inactivating enzyme from dog liver. One mole of inorganic phosphate per 124,000 gm. of phosphorylase was liberated during the course of the inactivation, the appearance of inorganic phosphate paralleling the loss of phosphorylase activity. Further, liver slices were observed rapidly to incorporate P_i^{32} into phosphorylase. When the labeled enzyme was then inactivated, P_i^{32} appeared. Epinephrine accelerated the resynthesis of phosphorylase and the incorporation of P^{32} into the enzyme.

Cowgill & Cori (32) have found that fresh extracts of the muscle of active lobsters contain an inactive form of phosphorylase. On standing the phosphorylase *b* activity of the extracts increased and then decreased while the phosphorylase *a* activity increased steadily to a final value five or six times

that of the initial extract. Dialysis of the extract permitted conversion of the inactive precursor to phosphorylase *b* while preventing its subsequent conversion to phosphorylase *a*. Versene inhibited the first step, indicating a requirement for a divalent cation. Conceivably the inactive precursor is identical with the product of the inactivation of phosphorylase by the liver inactivating system (31).

Some of the factors involved in conversion of phosphorylase *b* to *a* have been elucidated by Fischer & Krebs (33, 34) who noticed that, in contrast to previous reports, extracts of resting muscle contained mainly phosphorylase *b* rather than phosphorylase *a*. The addition of relatively small concentrations of divalent cations to such extracts sufficed to permit rapid conversion of phosphorylase *b* to *a*. Upon aging or dialysis, the crude muscle extracts developed a requirement for ATP for this transformation which was demonstrated to be catalyzed by a crude enzyme fraction from the fresh extracts. Although this fraction contained PR enzyme, purified PR enzyme did not itself catalyze conversion of phosphorylase *b* to *a* in the presence of divalent metal ions and ATP. Of a number of other nucleotides tested, only UTP showed any effect in activating the conversion, and this was slight in comparison with ATP.

These studies of the interconversion of phosphorylase *a* and *b* as well as their inactivation, the participation of uridine nucleotides in other oligosaccharide and mucopolysaccharide synthesis, and the report of Buell (35) that uridylic acid may be obtained after acid hydrolysis of crystalline phosphorylase indicate the possibility of uridine nucleotides in the structure and function of glycogen phosphorylase. Such a possibility has been suggested by Kalckar (36) while Calvin and his collaborators had earlier suggested that UDPG might serve as a general agent for glycosyl transfers although they did not specifically include glycogen phosphorylase (37). The known facts concerning the structure and function of phosphorylase may be encompassed in the hypothesis suggested below, in which phosphorylase *a* is considered to be a dimer united through a uridine dinucleotide which may also require the presence of a divalent cation. Fischer & Krebs have suggested such a structure without specifying the nature of the dinucleotide. Such a structure is analogous to that proposed for an actin dimer by Tsao (38).

It is suggested here that the substrate reacts with the enzyme, thereby cleaving the dimer; the resultant enzyme-UDPG complex then serves to carry out the desired polysaccharide synthesis while the enzyme-UMP residue is inert and awaits recombination with the other moiety to reconstitute the initial enzyme.

In these sequences, the following abbreviations are used: En and En′ are the two protein moieties of the dimer, UMP and PMU are uridylic acid, AMP and PMA are adenylic acid, UR is uridine, UDPG is uridine diphosphoglucose, G-1-P is glucose-1-phosphate, ATP is adenosine triphosphate, and P_i is inorganic phosphate.

Action of Phosphorylase *a* (M 495,000)

$$\text{En-UMP-PMU-EN}' + \text{G-1-P} \rightleftharpoons \text{En-UDPG} + \text{En}'\text{-UMP} \quad 1.$$

$$\text{En-UDPG} + \text{Primer} \rightleftharpoons \text{En-UDP} + \text{Primer-G} \quad 2.$$

$$\text{En-UDP} + \text{En}'\text{-UMP} \rightleftharpoons \text{En-UMP-PMU-En}' + P_i \quad 3.$$

$$\text{Over-all: G-1-P} + \text{Primer} \rightleftharpoons \text{Primer-G} + P_i$$

Phosphorylase *a* → Phosphorylase *b*

$$\text{En-UMP-PMU-En} \xrightarrow{H_2O} \text{En-UDP} + \text{En}'\text{-UR} \quad 4.$$

Action of Phosphorylase *b*

$$\text{En-UDP} + \text{AMP} \rightleftharpoons \text{En-UMP-PMA} + P_i \quad 5.$$

$$\text{En-UMP-PMA} + \text{G-1-P} \rightleftharpoons \text{En-UDPG} + \text{AMP} \quad 6.$$

$$\text{En-UDPG} + \text{Primer} \rightleftharpoons \text{En-UDP} + \text{G-Primer} \quad 2.$$

$$\text{Over-all: G-1-P} + \text{Primer} \rightleftharpoons \text{G-Primer} + P_i$$

Inactivation of Phosphorylase *b*

$$\text{En-UDP} \xrightarrow{H_2O} \text{En-UMP} + P_i \quad 7.$$

Inactivation of Phosphorylase *a*

$$\text{En-UMP-PMU-En}' \xrightarrow{H_2O} \text{En}'\text{-UR} + \text{En-UDP} \quad 4.$$

$$\text{En-UDP} \xrightarrow{H_2O} \text{En-UMP} + P_i \quad 8.$$

$$\text{Over-all: En-UMP-PMU-En}' \xrightarrow{H_2O} \text{En-UMP} + \text{En}'\text{-UR} + P_i$$

Phosphorylase *b* → Phosphorylase *a*

$$\text{En-UDP} + \text{En}'\text{-UR} + \text{ATP} \xrightarrow{Me^{++}} \text{En-UDP} + \text{En}'\text{-UMP} + \text{ADP} \quad 9.$$

$$\text{En-UDP} + \text{En}'\text{-UMP} \xrightarrow{Me^{++}} \text{En-UMP-PMU-En}' + P_i \quad 3.$$

Epinephrine Reactivation

$$\text{En}'\text{-UR} + \text{ATP} \rightarrow \text{En}'\text{-UMP} \quad 10.$$

$$\text{En}'\text{-UMP} + \text{ATP} \rightarrow \text{En}'\text{-UDP} \quad 11.$$

$$\text{En}'\text{-UDP} + \text{En-UMP} \rightleftharpoons \text{En-UMP-PMU-En}' + P_i \quad 3.$$

It will be seen that the hypothesis shown accounts for the halving of the molecular weight of phosphorylase *a* when converted to phosphorylase *b*; it provides a mechanism for reconversion of phosphorylase *b* to *a* involving nucleotide phosphorylation requiring ATP; reactivation of phosphorylase, inactivated by the liver enzyme, would give rise to P^{32}-labeled phosphorylase from P_i^{32} which was first incorporated into ATP by the liver system. As shown in reactions 1 to 3 an explanation is provided for the failure of the enzyme to catalyze exchange of G-1-P with P_i^{32} in the absence of primer

(39) in contrast to the action of *Pseudomonas* sucrose phosphorylase (40). To be sure, this speculation has no direct supporting evidence, but its attractive features warrant further investigation. No explanation is provided for the binding of 4 moles of adenylic acid per mole of phosphorylase *a* (41) nor is the nature of the linkage between the protein and the nucleotide specified. However, if the hypothesis be valid, the uridine nucleotides must be essentially nondissociable, since Kalckar (36) was unable to effect glycogen synthesis when UDPG was added to phosphorylase *a* and primer. In this relation the recent findings of Madsen & Cori are of interest (42). These authors found that phosphorylase *a* reacts with *p*-chloromercuribenzoate (PCMB), the extent of the reaction indicating 18.5 sulfhydryl groups per mole which agrees very well with a computed figure of a maximum of 18 sulfhydryl groups per mole. The inhibition of the phosphorylase activity paralleled the extent of interaction between PCMB and sulfhydryl groups, the enzyme being completely inhibited after the addition of 17 moles of PCMB per mole of enzyme. Sedimentation data indicate that phosphorylase *a* (M 480,000) was cleaved into four subunits (M 120,000) by the addition of PCMB while phosphorylase *b* (M 240,000) was cleaved into two similar subunits by this reagent. Addition of cysteine reconverted the phosphorylase subunits to phosphorylase *a* while addition of cysteine to the subunits from phosphorylase *b* gave rise once again to phosphorylase *b*. Detailed studies of the interaction of phosphorylase *a* and *b* with protamines (43, 44) and with detergents (45) have been reported.

An elegant study of glycogen "regeneration" *in vivo* has been reported by Stetten & Stetten (46). Glucose-C^{14} was given to rats and glycogen obtained from liver and carcass at various times thereafter. The glycogen was degraded by successive alternate treatments with muscle phosphorylase and amylo-1,6-glucosidase. The specific activity of the glucose released from each tier was then determined. At 6 hr., the relative activity of the outer tiers of liver glycogen was several times that of the center of the molecule; after 48 hr. this situation was reversed. In contrast, after 24 hr., the relative activity of the outer tiers of muscle glycogen was still twice that of the inner tiers. The authors interpret these findings as indicating that branches with glucose-C^{14}, initially in the periphery, by the action of branching enzyme [amylo-(1,4→1,6)-transglucosidase] are transferred to the interior of the molecule and that this process is considerably more active in liver than in muscle. They did not discuss the possibility that the observed difference between the behavior of liver and muscle glycogen may reflect a more dynamic turnover of the glucose, and glycogen, in liver than in muscle with equal "branching enzyme" activity in both tissues. The rapidly reduced relative specific activity of the outer tiers of liver glycogen would then result from dilution by fresh, unlabeled glucose. This is compatible with the fact that the absolute specific activity of liver glycogen was much more markedly reduced with the passage of time than was that of muscle glycogen.

Larner (47) has demonstrated that the action of phosphorylase in de-

grading glycogen is, at least partially, multichain in nature, by determining the yield of limit dextrin (assayed by the free glucose liberated upon subsequent treatment with anylo-1,6-glucosidase) at varying time intervals. A similar conclusion was reached by Whelan & Bailey (48) with respect to the synthetic action pattern of potato phosphorylase with maltotriose or maltotetrose as primers.

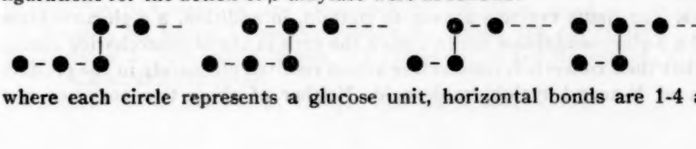
Transglycosidation.—The dextran synthesizing enzymes of a number of microorganisms have been subjected to further study. Bailey *et al.* (49) found that the product of dextransucrase activity in growing cultures of *Betacoccus arabinosaceus* is dependent upon the other components of the medium. Thus, the presence of glucose in quantity resulted in the accumulation of isomaltose and isomaltotriose. Addition of maltose resulted in panose accumulation as well as the appearance of a number of di- and trisaccharides containing a ketose sugar. Free fructose yielded a disaccharide containing the ketose also while α-methyl glucoside yielded oligosaccharides containing the methyl glycosylglucoside moiety. In general a high concentration of acceptor monosaccharide suppressed the formation of polymeric dextrans in accordance with numerous previous observations and suggesting a "multichain" mechanism for the transferring enzyme. Essentially similar observations have been reported with *Leuconostoc mesenteroides*, where the presence of maltose in the reaction mixture led to the synthesis of oligosaccharides and low molecular weight dextrans by dextransucrase (50). A transglucosidase from *Penicillium chrysogenum* Q-176 has been identified (51) which, with maltose as substrate, yielded isomaltose, isomaltotriose, panose, and 4-α-isomaltotriosyl-D-glucose as well as some higher oligosaccharides. Thus the enzyme appears to be an amylo-1,6-transglycosidase which yields no products which may be split by β-amylase or serve as primers for phosphorylase, indicating that there are no successive pairs of 1-4 linkages. The transferring enzyme from *Aspergillus oryzae* (52) appears to have similar properties. Originally observed to convert maltose to dextran, it works equally well with isomaltose, dextrotriose, and panose as substrates and exchanged glucose-C^{14} among them. Thus, it is a reversible transferring system which does not catalyze hydrolysis under known circumstances. The partial purification of a plant transfructosidase which builds up glucofructosans from sucrose with the simultaneous release of free glucose has been described (53). Under the conditions of study the chief product appeared to be fructosylfructosyl glucose. Small amounts of free fructose appeared during the incubation indicating that the enzyme has hydrolytic properties or perhaps that it had not been freed from a contaminating fructosidase.

Glycosidases.—Koshland, continuing his investigations of the mechanism of enzyme action (54), has now offered evidence supporting the generalization that enzymes catalyzing the reaction $ROQ + R'OH \rightarrow ROR' + QOH$, and showing high specificity for R but low specificity for Q cause rupture of the R-O bond. Thus, β-glucosidase which is specific for the glucose moiety, but not for the aglycone, should split salicin between the glucose carbon and

the bridge oxygen. When the enzymatic hydrolysis was conducted in H_2O^{18}, the C-1 position of the glucose contained the same O^{18} concentration as that of the medium while the salicin contained no detectable O^{18}. This is consistent with a displacement on the C-1 of the glucose and is support for the generalization previously proposed. Further, it was found that then free glucose was incubated in H_2O^{18} with β-glucosidase, the glucose reisolated contained amounts of O^{18} which were proportional to the enzyme concentration.

Several additional instances have been reported (55) of anomeric inversion during glycosidase action similar to that previously established for β-amylase. Thus, maltose yielded β-glucose under the influence of takadiastase maltase but not with pancreatic maltase, indicating a fundamental difference in the nature of the catalytic process with these two maltases. Similarly, cyclohexaglucan yielded a β-compound as indicated by upward mutarotation under the influence of the takadiastase mixture of enzymes. Upward mutarotation was also observed during the hydrolysis of cellopentose by the cellulase of *Myrothecium verrucaria* (56), indicating that the reducing groups were liberated in the β-configuration. In a brief report Larner & Gillespie (57) have summarized evidence indicating that histidine, in the protonated form, is the catalytic locus in intestinal maltase and oligo-1, 6-glucosidase (55). Similar suggestions have been made before for other hydrolytic enzymes including choline esterase, chymotrypsin, ribonuclease, lysozyme, amylo-1,6-glucosidase, and histidase. The mechanism suggested by the authors includes (*a*) formation of an oxonium cation of the acetal oxygen with the histidine proton, (*b*) displacement by a neighboring group with possible intermediate formation of a new acetal linkage between glucose and enzyme, and (*c*) hydrolysis and regeneration of the displacing group.

Two studies have been reported of the specificity of action of β-amylase. Whelan & Roberts (58) observed that the enzyme degraded even numbered maltodextrins completely to maltose; it very slowly yielded glucose and maltose from maltotriose while it degraded other odd numbered maltodextrins to maltose and maltotriose liberating little or no glucose during this process. Sumner & French (59) studied the action of crystalline β-amylase from sweet potatoes on low molecular weight branched oligosaccharides of known structure prepared by the coupling reaction catalyzed by macerans amylase. One series was obtained by the coupling of panose and α-Schardinger dextrin and another series from isomaltose and Schardinger dextrin. The oligosaccharide mixtures were separated by paper chromatography and then subjected to the action of β-amylase. In addition a branched heptasaccharide of known structure, obtained by action of salivary amylase on amylopectin, was used as substrate. The results indicated that the limiting terminal branch configurations for the action of β-amylase were as follows:



where each circle represents a glucose unit, horizontal bonds are 1-4 and

vertical bonds 1-6. A detailed report of this work will be awaited with interest.

Pazur & Budovich (60) followed the hydrolysis of amylotriose, labeled with C^{14} in the reducing position, by crystalline salivary amylase and found that the glucose and maltose liberated were approximately equally labeled indicating that the enzyme had equal access to the two glycosidic bonds in the substrate. The 1-C^{14}-amylotriose which was used as substrate in these studies was prepared from cyclohexamylose and glucose-1-C^{14} by the coupling action of macerans amylase and the hydrolytic action of β-amylase and isolated by mass paper chromatography (61).

Larner & McNickle (62) have partially purified from intestinal mucosa an enzyme, which they have called oligo-1,6-glucosidase, which hydrolyzes isomaltose, panose, and the α-amylase dextrins. The same enzyme has also been noted by Seiji (63). The new enzyme is differentiated from amylo-1,6-glucosidase since the latter has no activity with isomaltose, panose, or α-amylase dextrins as substrates, whereas it does remove sugar from the phosphorylase and β-amylase limit dextrins. Thus the digestion of starch and glycogen in the gastrointestinal tract is accomplished by the concerted action of salivary and pancreatic α-amylases, maltase, and the new oligo-1, 6-glucosidase. According to Wetter (64) the limit dextrinase of *Aspergillus niger* is incapable of hydrolyzing isomaltose, thereby indicating that the enzyme resembles amylo-1,6-glycosidase rather than oligo-1,6-glycosidase. The data also suggest, surprisingly, that isomaltose may be hydrolyzed by aspergillus maltase although without convincing evidence.

A new transglycosidase has been observed in the mycelium in cell-free extracts of the mycelium of *Chaetomium globosum* which cause the dissimilation of cellubiose into glucose and a trisaccharide whose linkages are both beta-1:4 (65).

Hash & King (66) have suggested that the appearance of a tetrasaccharide in the dialysate, when cellulose is incubated with the culture filtrate from *Myrothecium verrucaria* in a dialysis bag, indicates that the cellulase attacks the cellulose in random fashion. The tetrose was converted to cellobiose and glucose upon further action by the enzyme without the transient appearance of a cellotriose according to the paper chromatography system used.

Hyaluronidase and related enzymes.—Linker, Meyer & Weissmann (67) have studied the degradation of hyaluronic acid by the concerted action of the enzymatic mixtures in liver and testes extracts. Hyaluronidase is an endohexosaminidase in that it causes hydrolysis of N-acetylglucosaminidic bonds in the interior of the polysaccharide molecule in a manner similar to that of α-amylases digesting starch or glycogen. This results in rapid "liquefaction" and the accumulation of a mixture of even-numbered oligosaccharides. The tissue extracts appear to contain, in addition, a β-glucuronidase and a β-glucosaminidase which attack the ends of the oligosaccharide chains so that their concerted, consecutive action resulted ultimately in the production of N-acetyl-hyalobiuronic acid. Neither of these two enzymes was

purified, but data are presented which make it unlikely that the glucosaminidase is identical with that of earlier authors.

Weissmann (68) has found that testes hyaluronidase acting on oligosaccharides built of 2 to 6 units of N-acetyl hyalobiuronic acid catalyzes transglycosidation more rapidly than it does hydrolysis. There were indications that when the enzyme is acting on the longer chained substrates, the unit moved is a tetrasaccharide rather than a disaccharide. Schulte & Greiling (69) have reported that the oligosaccharides obtained by the action of pneumococcal hyaluronidase on hyaluronic acid are not identical with those obtained by the action of testes hyaluronidase in their behavior in several paper chromatography systems, although the smallest oligosaccharides reported behaved on hydrolysis as if they were either a disaccharide of acetyl glucosamine and glucuronic acid or the corresponding tetrasaccharide. No explanation is offered for the discrepant behavior in the paper chromatographic system. This material strongly inhibits testicular hyaluronidase, and its presence is a matter of considerable interest (70).

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II. GLYCOLYSIS

Occurrence and role of Embden-Meyerhof pathway.—The evidence is overwhelming that enzymes of the Embden-Meyerhof scheme occur in most, if not all bacterial, plant, and animal cells. Recent papers supporting this view are listed in the bibliography (1, 2, 3).

Of particular interest is the finding (4, 5) that certain obligate aerobes contain all of the enzymes required for pyruvic acid formation from glucose via the Embden-Meyerhof scheme but lack enzymes for the anaerobic disposal of pyruvate (carboxylase, DPN-linked lactic dehydrogenase). This not only illustrates the nature of the enzymatic defect responsible for the strict dependence of these organisms on oxygen for growth, but also suggests that the "glycolytic" enzymes leading to pyruvate play an important role in normal oxidative metabolism in these nonglycolyzing organisms.

The new well-established alternative pathway of hexose metabolism via pentose is described in another section of this chapter. An interesting example of the cooperative functioning of the Embden-Meyerhof scheme and the alternative pathway has been described by Gibbs *et al.* (6), who found through studies with ribose-1-C^{14} that ribose fermentation to ethanol and CO_2 in yeast proceeds in two stages. The first stage involves hexosephosphate formation via the alternative pathway; the second stage involves hexosephosphate fermentation via the Embden-Meyerhof scheme.

Site of insulin action.—Three major questions remain unsatisfactorily answered: (*a*) Does insulin act primarily on the transfer of glucose across the cell membrane, or is its main function to promote glucose utilization within the cell? (*b*) Which, if any, of the known enzymatic reactions of carbohydrate metabolism is influenced directly by insulin? (*c*) Does insulin act primarily on peripheral tissues (muscle) or does it also have a direct action on the liver?

Concerning the first question, Park *et al.* (7) have presented evidence that insulin addition *in vitro* to rat diaphragm muscle bathed in glucose solution leads to an accumulation of free glucose within the cells. This evidence has been strengthened by subsequent *in vivo* experiments by Park & Johnson (8) demonstrating a similar effect of injected insulin on the glucose content of the diaphragm and other muscles of the rat under appropriate conditions.

These experiments give strong support to the view of Levene & Goldstein (9) that insulin promotes glucose transport.

On the other hand, the new experiments of Burk and his collaborators (10, 11) argue for an effect of insulin on glucose utilization rather than on transport. Their experiments were carried out with cell-free homogenates of tumor tissue from rats. Homogenates prepared from stressed animals showed a greatly impaired ability to ferment glucose to lactic acid, but this ability could be restored to normal by *in vitro* addition of insulin. These experiments confirm strikingly and extend the observations of Colowick, Cori & Slein (12), who had found that when the hexokinase reaction in cell-free brain extracts was inhibited by certain anterior pituitary fractions, the inhibition could be prevented by addition of insulin. That hexokinase was also the site of action of insulin in the experiments of Burk *et al.* is suggested, but of course by no means proven, by the fact that the homogenates from stressed animals showed undiminished ability to ferment hexosediphosphate.

The action of insulin on glucose transport across the cell membrane is not incompatible with the view that insulin acts on hexokinase, since the latter may very well be concerned with the transport process. However, in view of the absence of glucose-6-phosphatase from muscle, it is difficult to visualize how hexokinase participation in the transport process could result in an increased free glucose concentration within the cell.

Concerning the question of the tissue site of insulin action, Renold *et al.* (13) report that insulin injection into diabetic animals affects the metabolism in diaphragm muscle within minutes, but affects the metabolism in liver only after many hours. These authors could show no consistent effect of insulin added to diabetic liver slices *in vitro* and concluded that the effects of insulin on the diabetic liver *in vivo* could be regarded as a secondary consequence of the peripheral action of insulin. There can be no question, however, that insulin does exert a direct effect on the metabolism of normal liver, as can be demonstrated by *in vitro* experiments. One may speculate that the liver enzyme which is normally directly subject to insulin action disappears from the liver during the diabetic state, whereas the corresponding muscle enzyme is "constitutive."

The content of glucose-6-phosphatase in rat liver increases during diabetes and can be restored to normal by insulin injection, but not by *in vitro* addition of the hormone (14, 15). This enzyme does not therefore appear to be directly under the influence of insulin. Its elevation would account for the excessive production of glucose by the liver in diabetes. Furthermore, since glucose-6-phosphate may play a key role in furnishing the reduced TPN which is necessary, according to Langdon (16), for the final reductive step in fat synthesis, an elevated glucose-6-phosphatase could help to account for the impaired fat synthesis in diabetic liver. Similar interpretations may be applied to the increase in liver glucose-6-phosphatase on fasting (17). One must emphasize again, however, that muscle does not contain glucose-6-phosphatase, but nevertheless exhibits pronounced dependence on insulin for fat synthesis. In muscle the only enzyme systems which can regulate the

glucose-6-phosphate concentration are hexokinase and phosphohexoisomerase. In addition to the enzymes thus far mentioned, the "condensing enzyme" which makes citric acid has also been implicated as a possible site of insulin action (18).

Hexokinases.—The subject of substrate specificity of the hexokinases has become a matter of some importance with respect to elucidating the mechanism of certain physiological processes in mammals. Whereas *Schistosoma mansoni* has been shown to contain four separate kinases acting on glucose, fructose, mannose, and glucosamine (19, 20), this is not so for mammalian tissues (21) and yeast (22), which contain a single hexokinase acting on all four sugars. Bhattacharya (23) has made ingenious use of the latter observation to support his view that alloxan produces diabetes by inhibiting the hexokinase of the islet cells of the pancreas; he has shown that various injected sugars are able to protect animals from the diabetogenic action of alloxan and that this ability is related directly to their affinity as substrates for mammalian hexokinase.

The substrate specificity of intestinal hexokinase is reported by Sols (24) to be similar to that of the brain enzyme (21); correlation with the substrate specificity for intestinal absorption of sugars leads him to conclude that the latter process does not involve phosphorylation.

The mechanisms of action of the hexokinase of yeast have been studied with isotopically labelled substrates. Gamble & Najjar (25), using C^{14}-labelled glucose and unlabelled glucose-6-phosphate, showed that no label is introduced into the latter by the enzyme unless small amounts of ADP (or ATP) are added. Similarly, Kaufman (26) has shown that P^{32}-labelled ADP is not incorporated into ATP by the enzyme unless glucose is added. These experiments indicate that neither of the following reactions occurs reversibly to a measurable extent:

$$\text{ATP} + \text{enzyme} \rightleftharpoons \text{P-enzyme} + \text{ADP} \qquad 1.$$

$$\text{P-enzyme} + \text{glucose} \rightleftharpoons \text{enzyme} + \text{G-6-P} \qquad 2.$$

but that the over-all reaction:

$$\text{ATP} + \text{glucose} \rightleftharpoons \text{ADP} + \text{G-6-P} \qquad 3.$$

is surprisingly readily reversible. A phospho-enzyme intermediate may therefore be ruled out for the case of yeast hexokinase. Corresponding experiments with the mammalian enzyme have not been reported but might prove interesting in view of its greater susceptibility to inhibition by G-6-P (27).

The subject of naturally-occurring inhibitors and activators of hexokinase has received considerable attention during the 10 years since the first announcement (12, 28) of an insulin-reversible inhibition of mammalian hexokinase by anterior pituitary extracts. According to Stern (29) the apparent inhibition of hexokinase by nerve and placental extracts is entirely accounted for by their adenosinetriphosphatase activity. He confirms the presence of a true inhibitor of hexokinase in dialyzed plasma, but this inhibition is not insulin-reversible. Bornstein & Krahl (30, 31) have reported an insulin-re-

versible inhibition of glucose uptake in intact diaphragm muscle by a lipoprotein fraction from anterior pituitary extracts, but this fraction has no effect on cell-free hexokinase preparations. The activation of hexokinase by factors in muscle and in hemolysates is explained, according to Long & Thomson (32), by their content of phosphohexokinase, which aids in the removal of inhibitory hexosemonophosphate. However, Stern (29) regards this explanation as insufficient.

Several new synthetic inhibitors of hexokinase have been described, including N-lauroyl sarcosinate (33) and N-acyl derivatives of D-glucosamine (34). The latter are particularly interesting, since they are likely to be highly specific and are inhibitory in concentrations as low as 10^{-6} *M*. Inhibitory effects of salts (34a), anions (35), and cations (36) on hexokinase have also been described.

Another inhibitor, 6-deoxy-6-fluoro-D-glucose, produces much more marked effects on glucose fermentation by intact yeast than it does on glucose fermentation by yeast extracts or on yeast hexokinase (37). This and other observations have led to the postulate that the inhibitor acts on a specific process, not limited by hexokinase activity, which controls the rate of entry of glucose into the yeast cell. The same inhibitor had much less effect on glucose uptake by rat diaphragm.

Galactose metabolism.—The failure to metabolize galactose is an inherited defect in humans. Schwarz & Goldberg (38) have shown that there is an accumulation of galactose-1-phosphate in the lens of galactosemic individuals. Such individuals have been shown recently by Isselbacher *et al.* (39) to lack the uridyl transferase which catalyzes the reaction, galactose-1-phosphate+UDPG$\rightleftharpoons$glucose-1-P+UDPGal.

The conversion of UDPGal to UDPG by the enzyme galactowaldenase requires DPN (40), suggesting that the process involved is oxidation-reduction rather than Walden inversion. Studies on genetic control of galactose metabolism in yeast (41) suggest that separate genes are concerned in the entry of galactose into the cell and in the subsequent conversion of galactose to glucose-6-P.

Fructose and hexitol metabolism.—The scheme of Hers (42) for the conversion of fructose to liver glycogen via triose phosphate has been confirmed by isotope experiments which show that administered fructose-1-C^{14} gives rise to liver glycogen containing glucose highly labelled in carbons 1 and 6. Glucose-1-C^{14}, on the other hand, gives rise to glycogen containing glucose labelled almost exclusively in carbon 1, confirming the view that fructose is converted directly to trioses. When sorbitol-1-C^{14} was injected, the labelling of the liver glycogen was the same as when fructose-1-C^{14} was injected, proving that sorbitol gives rise to fructose rather than glucose in the intact rat. Similar conclusions have been reached from studies on infusion of sorbitol into rats (43). In certain bacteria, sorbitol gives rise to sorbose (44) and mannitol-6-phosphate is oxidized to fructose-6-phosphate (45).

The cleavage of both fructose-1-phosphate and fructose-1,6-diphosphate is catalyzed by the "1-phosphofructaldolase" of liver, whereas highly puri-

fied muscle aldolase cleaves only fructose-1,6-diphosphate, according to Leuthardt & Wolf (46). They attribute earlier reports of some fructose-1-phosphate cleavage by crystalline muscle aldolase to the presence of a 1-phosphofructaldolase of muscle origin in the test system. They report further that the liver preparation is capable of synthesizing large amounts of xylulose-1-phosphate, ketohexose-1-phosphate and fructose-1-phosphate from fructose-1,6-diphosphate and the appropriate nonphosphorylated aldehyde whereas the crystalline muscle aldolase is much less effective. While this may reflect simply a broader specificity of the liver enzyme, one must also keep in mind the fact that this enzyme preparation contains triosephosphate isomerase, whereas the pure muscle aldolase does not. It may be calculated that the concentration of dihydroxyacetone phosphate at equilibrium in the liver aldolase-isomerase system would be seven to eight times as high as in the pure muscle aldolase system, and this might very well account for the differences observed in the yields of ketose-1-phosphate in the liver and muscle systems.

The rate of synthesis of ketose-1-phosphates by the muscle system, while low compared with the liver system, is nevertheless surprisingly high when one recalls that the pure muscle enzyme fails to cleave ketose-1-phosphates at all. Leuthardt & Wolf suggest that this may simply reflect (*a*) the fact that equilibrium is highly in favor of ketohexose-1-phosphate synthesis or (*b*) the possible existence of an intermediate dihydroxyacetone phosphate compound with the enzyme. If such a compound were formed, ketose-1-phosphate synthesis could occur rapidly by a transfer of the dihydroxyacetone phosphate group to the free aldehyde, without requiring the presumably slower formation of free dihydroxyacetone phosphate. As a test of the latter hypothesis, it would be of interest to study the ability of fructose-1-phosphate to serve as donor of the dihydroxyacetone phosphate group in the muscle aldolase system, using glycol aldehyde as the acceptor and measuring ketopentose-1-phosphate. If the group-transfer view is correct, fructose-1-phosphate, though not cleaved appreciably, might nevertheless participate measurably in group transfer.

Direct evidence for the formation of a compound of muscle aldolase with dihydroxyacetone phosphate has been obtained by Rose & Rieder (47), who demonstrated by tritium exchange reaction 4. The existence of such a com-

$$\begin{array}{l} CH_2OP \\ | \\ C{=}O \\ | \\ CH_2OH \end{array} + \text{aldolase} \rightleftharpoons \begin{array}{l} CH_2OP \\ | \\ C{=}O \\ | \\ CHOH \\ | \\ \text{aldolase} \end{array} + H^+ \qquad 4.$$

pound creates the possibility of a more rapid exchange of carbons 4,5, and 6 than of 1, 2, and 3 into fructosediphosphate. Thus, the experiments of Eisenberg (48) in which methyl-labelled lactate gave rise to glucose with C_6/C_1 ratio of 1:1 to 1:2 could be explained by the following sequence:

Labelled lactate → labelled glyceraldehyde phosphate:

labelled glyceraldehyde phosphate + unlabelled fructose-diphosphate $\xrightarrow{\text{aldolase}}$ fructosediphosphate labelled at carbon 6.

Other explanations, involving washing out of label at carbon 1 by the shunt mechanism, must also be considered.

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III. THE SHUNT PATHWAY

Quantitative evaluation of the participation of the HMP pathway in metabolism.—At first glance it would seem that the important attempts to quantitate the proportional contribution of the EM path and the HMP path in dissimilation of glucose are predicated on limiting assumptions, which, for the sake of simplicity, may fail to describe adequately the true sequence of events. Aside from the problem of the fate of two-carbon fragments which may leave the HMP path and appear in products common to both pathways (CO_2, acetate, alcohol, lactate) for which no allowance can accurately be made because of lack of knowledge of the nature and size of pools through which these fragments must pass, equations derived on the assumption of one turn of the HMP cycle will fail to account for isotope distribution into other carbons of glucose, and for corresponding dilution of the original labeled carbon, which are possible consequences of continouous operation of the HMP path. The problem is somewhat analogous to attempting to predict the labeling of glycogen from methyl-labeled pyruvate on the assumption that the TCA cycle may complete only one turn. Such an assumption precludes the possibility of 3,4 labeled glucose, which is contrary to experience, and indicates that such a limiting assumption is invalid.

The following sequence of events, which depends upon reactions known

to be catalyzed by enzymes of the H-P pathway, may serve to illustrate the possible extent of isotope wandering (nothing is implied concerning the rates of the steps indicated). The yields of various labeled hexose would obviously be dependent upon the relative rates of the indicated steps.

I.

$$\begin{array}{llll}
\mathrm{C1} & & & \\
\mathrm{C2} & & \mathrm{C1} & \\
\bar{\mathrm{C}}3^{-} + & & \rightarrow \mathrm{C2} & + \mathrm{C3} \\
\mathrm{C4} & \mathrm{C4} & \mathrm{C4} & \mathrm{C4} \\
\mathrm{C5} & \mathrm{C5} & \mathrm{C5} & \mathrm{C5} \\
\mathrm{C6} & \mathrm{C6} & \mathrm{C6} & \mathrm{C6}
\end{array}$$

$$C_6P + C_3P \rightarrow C_5P + C_4P$$

II.

$$\begin{array}{llll}
 & & \mathrm{C1} & \\
\mathrm{C1} & & \mathrm{C2} & \\
\mathrm{C2} & \mathrm{C1} & \mathrm{C1} & \\
\bar{\mathrm{C}}3^{-} & \mathrm{C2} & \mathrm{C2} & \mathrm{C3} \\
\mathrm{C4} & \mathrm{C4} \rightarrow & \mathrm{C4} + & \mathrm{C4} \\
\mathrm{C5} & \mathrm{C5} & \mathrm{C5} & \mathrm{C5} \\
\mathrm{C6} & \mathrm{C6} & \mathrm{C6} & \mathrm{C6}
\end{array}$$

$$C_6P + C_5P \rightarrow C_7P + C_4P$$

III.

$$\begin{array}{lll}
\mathrm{C1} & & \\
\bar{\mathrm{C}}2^{-} & \mathrm{C2} & \\
\mathrm{C3} & \mathrm{C3} & \\
3\ \mathrm{C4} \rightarrow & 3\ \mathrm{C4} & + 3\ \mathrm{CO_2} \\
\mathrm{C5} & \mathrm{C5} & \\
\mathrm{C6} & \mathrm{C6} &
\end{array}$$

$$3\ C_6P \rightarrow 3\ C_5P + 3\ CO_2$$

IV.

$$\begin{array}{llll}
 & & \mathrm{C2} & \\
\mathrm{C2} & & \mathrm{C3} & \\
\mathrm{C3} & \mathrm{C3} & \mathrm{C3} & \\
2\ \bar{\mathrm{C}}4^{-} + & 2\ \mathrm{C4} \rightarrow & 2\ \mathrm{C4} & + 2\ \mathrm{C4} \\
\mathrm{C5} & \mathrm{C5} & \mathrm{C5} & \mathrm{C5} \\
\mathrm{C6} & \mathrm{C6} & \mathrm{C6} & \mathrm{C6}
\end{array}$$

$$2\ C_5P + 2\ C_4P \rightarrow 2\ C_6P + 2\ C_3P$$

V.

$$\begin{array}{llll}
\mathrm{C1} & & & \\
\mathrm{C2} & & \mathrm{C1} & \\
\mathrm{C1} & & \mathrm{C2} & \\
\bar{\mathrm{C}}2^{-} & & \mathrm{C1} & \mathrm{C2} \\
\mathrm{C4} & + \mathrm{C4} \rightarrow & \mathrm{C4} & + \mathrm{C4} \\
\mathrm{C5} & \mathrm{C5} & \mathrm{C5} & \mathrm{C5} \\
\mathrm{C6} & \mathrm{C6} & \mathrm{C6} & \mathrm{C6}
\end{array}$$

$$C_7P + C_3P \rightarrow C_6P + C_4P$$

VI.

$$\begin{array}{llll}
 & & \mathrm{C2} & \\
\mathrm{C2} & & \mathrm{C3} & \\
\mathrm{C3} & \mathrm{C2} & \mathrm{C2} & \\
\bar{\mathrm{C}}4^{-} + & \mathrm{C4} \rightarrow & \mathrm{C4} & + \mathrm{C4} \\
\mathrm{C5} & \mathrm{C5} & \mathrm{C5} & \mathrm{C5} \\
\mathrm{C6} & \mathrm{C6} & \mathrm{C6} & \mathrm{C6}
\end{array}$$

$$C_5P + C_4P \rightarrow C_6P + C_3P$$

Sum:

$$\begin{array}{lll}
 & 1 & 2212 \\
 & 2 & 3323 \\
 & 3 & 3312 \\
5\ C_6P & 4 \rightarrow 4\ C_6P & 4444 + C_3P + 3\ CO_2 \\
 & 5 & 5555 \\
 & 6 & 6666
\end{array}$$

To the extent that the indicated reactions are operative, for every molecule of glucose disappearing with the formation of triose and 3 CO_2, 4 molecules of glucose remaining will have been reshuffled so that isotope originally present in C_1 can now be found in C_3, isotope originally in C_2 in both C_1 and C_3, and isotope from C_3 is also present in C_2. Two turns of the indicated sequence will return hexose molecules in which isotope originally present in

any one of carbon atoms 1, 2 or 3 will have found its way into each of the other two. Isotope randomization would extend to carbons 4, 5, and 6 if triose phosphate isomerase, aldolase, and hexose diphosphatase were operative in the tissues studied. Since these enzymes are present in liver, this organ is potentially capable of producing CO_2 derived from any one of the six carbons of hexose without resort to the TCA cycle.

The considerations outlined above indicate that in systems possessing the requisite enzymes for the EM and HMP pathways, the specific activity of carbon dioxide derived from glucose labeled in any single position should vary with time in a manner dependent on the relative rates of the reactions involved, so that no single measurement at an arbitrary time should be capable of providing definite information concerning the pathway. Extrapolation of specific activity data to zero time might, however, provide an appropriate measure.

Assuming no randomization of isotope extending to carbon 4, 5, and 6 of the type discussed above, and an equal rate of formation of CO_2 from C_1, C_2, and C_3, one can calculate the participation of the shunt pathway as follows:

A = specific activity of glucose (counts/min./μmole)

S = fraction of CO_2 via shunt pathway

G = fraction of CO_2 via glycolysis and TCA cycle = $1-S$

X_1 = specific activity of CO_2 from C_1 glucose (counts/min./μmole)

X_6 = specific activity of CO_2 from C_6 glucose (counts/min./μmole)

then, from C_1 labeled glucose:

(I) $$X_1 = \frac{AS}{3} + \frac{AG}{6} = A\left(\frac{2S+1-S}{6}\right) = A\left(\frac{S+1}{6}\right)$$

$$S = \frac{6X_1}{A} - 1; \qquad G = 1 - S \qquad G = 2 - \frac{6X_1}{A}$$

from C_6 labeled glucose:

(II) $$X_6 = \frac{AG}{6} = \frac{A}{6}(1-S)$$

$$G = \frac{6X_6}{A} \qquad S = \frac{A - 6X_6}{A}$$

(III) $$\text{Let } \frac{X_6}{X_1} = R \text{ then}$$

$$R = \frac{\dfrac{A(1-S)}{6}}{\dfrac{A(1+S)}{1}} = \frac{1-S}{1+S}$$

$$S = \frac{1-R}{1+R}; \qquad G = \frac{2R}{1+R}$$

These equations should be valid even when the rate of CO_2 formation from C_2, C_3, C_4, and C_5 of glucose via the TCA cycle is much greater than from C_1 and C_6 since dilution of X_1 and X_6 should occur by the same factor and would not affect the ratio. The equations should apply to values extrapolated at zero time for specific activities.

If the oxidation of carbon 1 of glucose via the shunt pathway predominates over that of carbon 2 and 3 (e.g., because of a rapid utilization of ribose-5-phosphate for nucleotide synthesis) the specific activity of shunt CO_2 will approach A (specific activity of glucose) and the equations (I) to (III) will change to:

(Ia)
$$X_1 = AS + \frac{AG}{6} = A\left(\frac{6S + 1 - S}{6}\right) = A\left(\frac{5S + 1}{6}\right)$$

$$\frac{6X_1}{A} - 1 = 5S \qquad S = \frac{6X_1 - A}{5A}; \qquad G = 1 - S = \frac{6(A - X_1)}{5A}$$

(IIa)
$$X_6 = \frac{AG}{6} = \frac{A}{6}(1 - S)$$

$$G = \frac{6X_6}{A} \qquad S = \frac{A - 6X_6}{A}$$

(IIIa)
$$\text{Let } \frac{X_6}{X_1} = R \text{ then}$$

$$R = \frac{\frac{A}{6}(1 - S)}{\frac{A}{6}(5S - 1)} = \frac{1 - S}{5S + 1}$$

$$5SR + R = 1 - S$$

$$1 - R = 5SR + S$$

$$S = \frac{1 - R}{1 + 5R} \qquad G = \frac{6R}{1 + 5R}$$

HMP pathway in mammalian tissues.—Bloom *et al.* (1 to 3) have extended their studies on the participation of the shunt pathway in glucose utilization. Lactate-1-C^{14}, lactate-2-C^{14}, lactate-3-C^{14}, glucose-1-C^{14}, glucose-2-C^{14}, glucose-6-C^{14}, gluconate-1-C^{14}, gluconate-6-C^{14}, and ribose-1-C^{14} were used with rat liver slices. Two equations were derived to permit estimation of the fraction of glucose catabolized via glycolysis. Their findings on the distribution of label in CO_2, glycogen, and fatty acid are in accord with the presence of the shunt pathway in liver slices. The authors estimate that approximately one-half of the glucose is utilized through the shunt pathway in liver. Brain slices appear to catabolize glucose entirely via the EM pathway, while many other tissues, particularly testis and bone marrow, formed more CO_2 from carbon 1 of glucose than from carbon 6 under their experimental conditions. Katz *et al.* (4, 5) and Abraham *et al.* (6) have carried out similar studies. In rat mammary glands they find 10 to 15 times as much carbon dioxide derived from C_1 as from C_6, and fatty acid formation is considerably greater from C_6-glucose that from C_1-glucose. From the ex-

tent of incorporation of C_1 and C_6 into fat, these authors estimate that 60 per cent of the glucose molecules are utilized via the shunt pathway. These authors also indicate that their data with C_2-labeled glucose invalidate some of the equations used earlier since C_2/C_6 ratios of 10 were obtained, whereas the C-alpha/C-beta ratio for lactate is only about 1.5. The same discrepancy of C_2 utilization was noted in liver by Agranoff *et al.* (7). It is obvious therefore that the greater the relative contribution of the shunt pathway, the greater the utilization of the C_2 carbon of glucose via the shunt. Equations which ignore this are obviously invalid. The calculations of Abraham *et al.* (6) regarding a 60 per cent participation of the shunt pathway based on fat formation from C_1 and C_6 agree closely with calculations based on equations (Ia) and (IIIa) derived above

$$\left(S = \frac{1 - R}{1 + 5R}\right).$$

The values of 33 per cent CO_2 derived from C_1 and 3.5 per cent derived from C_6 yield a calculated value of 60 per cent for the participation of the shunt pathway.

Agranoff *et al.* (7) report data of the ratios of $C^{14}O_2$ yield from glucose labeled in 1, 2, and 6 position. For diaphragm and kidney, values for the C_6,C_1 ratio are approximately one: In normal liver slices a C_6/C_1 ratio of 0.33, a C_2/C_1 ratio of 0.66, and a C_2,C_1 ratio of 2 were obtained. Since the alpha and beta carbons of lactate yield CO_2 in liver at a ratio of 1.4, it can be stated that C_2 carbon of glucose is utilized for CO_2 production to a greater extent than would be predicted if C_2 failed to appear in any form except lactate. This, of course, argues for the removal of carbon 2 by enzymes of the shunt pathway. They further observed that in fasted animals the C_6/C_1 rose to 0.48 in 48 hr. and to 0.8 in 72 hr., indicating a smaller participation of the shunt mechanism in the fasted animals. The C_2/C_6 ratio in the liver of fasted animals is, in fact, below 1, which serves to accentuate the lack of validity of equations with simplified pathways. These findings regarding the effect of the nutritional status of the animal may cast some light on the conclusions of Bernstein *et al.* (8) that the HMP pathway plays but a small quantitative role in the utilization of glucose in liver. Glycogen from the liver of fasted rats given $NaHC^{14}O_3$ were found to have little radioactivity in positions other than carbons 3 and 4. The distribution of C^{14} in carbons 1 and 2 were in accord with the operation of the shunt pathway, C_2 being higher than C_1, but the activity in C_6 was much lower than expected. The high radioactivity in C_3 and C_4 compared to C_1, C_2, C_5, and C_6 may indeed be attributable to a lack of mixing of the HMP pools or attributable to a low shunt pathway activity, as suggested by Wood (9). The possibility of an exchange reaction between HMP and glycogen, which could rapidly dilute the C_3 and C_4 labeled HMP, and which would prevent the re-cycling of the isotope, should also be considered.

Kinoshito *et al.* (10, 11) have demonstrated the steps of the HMP path-

way in homogenates of corneal epithelium and have made estimates of the relative role of the shunt pathway in corneal epithelium from studies of isotope incorporation into lactate from C^{14} labeled glucose. Kelly *et al.* (12) have shown that guinea pig adrenals contain the enzymes of the shunt pathway. Of particular interest in connection with the C_{11} hydroxylation of steroids is the very high activity of TPN-linked glucose-6-phosphate dehydrogenase found in adrenal cortex.

Activity levels of both glucose-6-phosphate and 6-phosphogluconate dehydrogenase were found to be significantly reduced in the liver extracts of starved and alloxan diabetic rats, while thyroxine treatment resulted in an approximately twofold increase in the level of both dehydrogenases. The activity of the dehydrogenases was found to be higher in summer than in winter. Insulin, growth hormone, or thyroxine added *in vitro* had no effect on the activity of these enzymes (13). The utilization of C_1 and C_6 of gluconate in liver slices of starved and alloxan diabetic rats was either normal or augmented, while C_1, C_2 and C_3 of glucose was oxidized at a diminished rate (14).

In connection with the operation of the shunt pathway, it is of interest that ribulose and L-xylulose have been identified in normal human urine (15). About 1 mg. of ribulose and 4 mg. of xylulose were isolated per liter of urine. A considerable increase in L-xylulose excretion occurs in cases of essential pentosuria and in normal human subjects after feeding of D-glucuronolactone (16). The utilization of gluconate and 2-ketogluconate was investigated by Salmony & Whitehead (17). In kidney slices the rate of oxidation with gluconate was one-sixth that of glucose but homogenates were inactive.

Alternate pathways in microorganisms.—An attempt to quantitate the contribution of the shunt mechanism in yeast and bacteria was made by Blumenthal *et al.* and Lewis *et al.* (18, 19). To correct for endogenous unlabeled carbon in common pools with the fragments derived from glucose, these authors use uniformly labeled glucose in comparison to C_1 labeled glucose. It is assumed that triose phosphates, formed via the EM process, arise equally from carbon 1-3 and 4-6, whereas those derived via pentose pathways arise only from carbon 4-6. Experiments with C_1 labeled glucose provide data on the relative incorporation of this carbon into intermediates such as lactate, acetate, or ethanol, while the data with uniformly labeled glucose provide a correction factor for dilution. In keeping with the results of Koshland & Westheimer (20), it was found that in *Saccharomyces cerevisiae* 95 to 97 per cent of the glucose is used anaerobically via the EM pathway. Aerobically, however, the shunt mechanism contributed about 15 per cent as measured with glucose-1-C^{14} and about 4 per cent as measured with glucose-6-C^{14}. The discrepancy again points up the inability of the described schemes to account for the metabolic events that are occurring. In *Torula utilis*, which is a more aerobic organism, the anaerobic results were the same. The aerobic results indicated a greater participation of shunt

pathway. Here again a discrepancy is noted between the glucose-1-C^{14} data which indicate a 25 per cent participation of the shunt, and the glucose-6-C^{14} data which indicate only 13 per cent participation. The authors state that there is no reason why the shunt should not operate anaerobically, since oxidative reactions could be coupled with reductive reactions through common coenzymes. However, the scarcity of dehydrogenases that can utilize reduced TPN requires the presence of a mechanism such as TPN cytochrome-*c* reductase, which is limited to the aerobic oxidation chain, while DPNH-linked dehydrogenases are available for classical fermentation via the EM pathway. Weinhouse and his collaborators (18, 19) are aware of the possible error in these calculations, particularly with regard to the possibility of transfer of carbon atoms 1 and 2 from fructose-6-phosphate (21), so that randomization of the label may occur in the upper three carbon atoms. Data obtained by the same method with bacteria (19) indicate that in *Serratia marcescens* the major portion of glucose metabolism is utilized via the pentose pathway with a smaller participation of the EM pathway and the phosphogluconic fermentation of Entner and Doudoroff. In *Proteus vulgaris* the EM was the major pathway, accounting for over 90 per cent of the glucose metabolism in resting cells and for 65 per cent in growing cells. In *Echerichia coli* about two-thirds of the acetate formed in resting or growing cells arose via the EM pathway.

Time course studies on the $C^{14}O_2$ liberation from C_1, C_2, and C_6 labeled glucose in bakers' yeast (22) revealed that carbon dioxide was produced most rapidly from glucose-1-C^{14} during the first 7 hr. of aerobic growth, suggesting a relatively active pentose phosphate cycle. However, $C^{14}O_2$ production from glucose-2-C^{14} lagged considerably behind the corresponding CO_2 production from glucose-6-C^{14}. Therefore, the equations (Ia) and (IIIa) derived above should be applicable in this case.

Participation of the shunt pathway in microorganisms has been deduced from various types of experimental evidence, among which the utilization of C^{14}-1-glucose, the demonstration of enzymes of the pentose phosphate cycle and the accumulation of pentose and heptulose intermediates are the most widely used. Limited space permits only brief quotations of these interesting studies. In *Acetobacter suboxydans* (23 to 27), *Tilletia caries* (28), *Azotobacter vinelandii* (29, 30), *Corynebacterium creatinovorans* (31), *Pasteurella pestis* (32, 33), and *E. coli* (34), evidence for the shunt pathway has been obtained. From cultures of *Acetobacter acetosum* grown on glucose, the calcium salts of 2-keto-D-gluconic and tartronic acids were isolated. Of interest also are the observations on the formation of tartronic acid, arabinose, and ribulose when aqueous solutions of 2-keto-D-gluconate were boiled in an atmosphere of nitrogen in the presence of calcium hydroxide (35). Various bacteria could be adapted to grow on a medium containing 2-keto-D-gluconate (36) and were examined for the presence of a 2-ketogluconokinase. The enzyme was demonstrated in *Pseudomonas*, *Xanthomonas*, *Escherichia*, *Aerobacter* and several others, while *Agrobacterium*, *Corynebacterium* and several

moulds, although capable of oxidizing 2-ketogluconate, did not appear to catalyze its phosphorylation. The reactions of the Entner-Doudoroff pathway have been demonstrated in bacterial plant pathogens and legume bacteria (37) and in *A. vinelandii* (38). In *Azotobacter* part of the glyceraldehyde-3-phosphate which is formed by the cleavage of 6-phosphogluconate appears to recycle to glucose-6-phosphate through the action of aldolase, fructose diphosphatase, and phosphohexose isomerase. *Thiobacillus thioparus* (39) was shown to incorporate carbon dioxide in the presence of ribulose-diphosphate, a reaction that is discussed in another section of this review.

Enzymic reactions of the shunt pathways.—A new enzyme that hydrolyzes 6-phosphogluconolactone, the first product of glucose-6-phosphate oxidation, has been discovered in bacteria, yeast, and mammalian tissues (40). The enzyme from yeast has been purified 36-fold and requires divalent cations, such as Mg^{++}, Mn^{++}, or Co^{++} for activity. The enzyme is specific for lactones containing the same configuration as D-glucono-δ-lactone at carbon 1, 2, 3 and 4. Xylonolactone (which is a substrate for glucose dehydrogenase) is not split by the lactonase in spite of the fact that configuration of C_1, C_2 and C_3 is the same as in glucose.

The purification and crystallization of transketolase from bakers' yeast have been described and its requirement for thiamine pyrophosphate and Mg^{++} demonstrated (21). In the presence of the crystalline enzyme, several keto sugars, as well as hydroxypyruvate, act as donor substrates giving rise to an "active glycolaldehyde" which is condensed with an acceptor aldehyde to a new keto sugar. From two pentose phosphates, one heptulose phosphate and one triose phosphate are formed. The triose phosphate was identified as glyceraldehyde-3-phosphate. Fructose-6-phosphate is shown to be a donor of "active glycolaldehyde" giving rise to pentose phosphate in the presence of triose phosphate, and to heptulose phosphate in the presence of ribose-5-phosphate as acceptor aldehyde. The formation of fructose-6-phosphate from pentose phosphate is shown to be dependent on the participation of both transketolase and transaldolase, and, based on these findings, a glucose-6-phosphate oxidation cycle is proposed.

Xylulose-5-phosphate was identified as the true donor substrate for transketolase (41). Ribulose-5-phosphate is inactive as donor substrate with crystalline transketolase from yeast. The nonphosphorylated sugars, ribulose and xylulose, show the same reactivity with transketolase, xylulose serving as a donor substrate, ribulose not. Ribulose-5-phosphate can be converted into xylulose-5-phosphate by a very active enzyme found in rabbit muscle and in red blood cells (41, 42). A similar enzyme is also present in spleen extracts (43) and in *Pseudomonas hydrophila* (44).

Transaldolase, which catalyzes the formation of fructose-6-phosphate and tetrose phosphate from sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate has been purified about 400-fold from yeast (45). In the reaction a dihydroxyacetone group is transferred from sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate. No evidence for a prosthetic group or co-

factor has been found. In the presence of aldolase and transaldolase, sedoheptulose diphosphate is formed from sedoheptulose-7-phosphate and fructose diphosphate. The evidence for the formation of sedoheptulose diphosphate is as follows: The isolated product contains two phosphate groups, one of which is relatively labile to acid hydrolysis. It is cleaved by aldolase to yield 1 mole of dihydroxyacetone phosphate. The sugar has been identified by conversion to sedoheptulosan tetrabenzoate. Aldolase shows a high affinity for sedoheptulose diphosphate, and the rate of reaction with this substrate is about 60 per cent of that observed with fructose diphosphate. The tetrose phosphate formed by transaldolase action is suggested to be D-erythrose-4-phosphate on the basis of its participation in the enzyme reactions of the shunt pathway (46).

Synthetic D-erythrose-4-phosphate (47) has been shown to be a substrate for transaldolase as well as for transketolase (48). Tetrose phosphate, prepared by the interaction of fructose-6-phosphate and glyceraldehyde in the presence of transketolase, appears to have the same properties as the synthetic compound. Erythrulose-1-phosphate is formed from formaldehyde and dihydroxyacetone phosphate by a new aldolase enzyme which was purified 40-fold from rat liver. Periodic acid oxidation was used to establish the structure of the tetrose phosphate (49). A similar enzyme was shown to occur in plant extracts (50). The reaction may represent an important pathway for formaldehyde assimilation. A 3-ketopentose arising from ribose-5-phosphate in spleen extracts has been described (51). The authors make the interesting suggestion that this reaction provides a possible mechanism for the formation of L-xylulose from D-ribulose.

An adaptive enzyme which catalyzes the isomerization of D-xylose and D-xylulose has been demonstrated in cell-free extracts of *Pasteurella pestis* (52). In the absence of borate, equilibrium is reached when about 16 per cent xylulose is present; with borate, 60 to 65 per cent xylulose accumulates. The K_m of the enzyme for D-xylose is about 3×10^{-3} *M* at pH 7.5. Tris (hydroxymethyl)-aminomethane inhibits the reaction in a noncompetitive manner. A xylulokinase is found in the crude extract, the enzyme phosphorylates D-xylulose in the presence of ATP, but not D-xylose. A ribose kinase was obtained from *E. coli* grown on ribose and was used for a large scale preparation of ribose-5-phosphate (53). A 2-ketogluconokinase was partially purified from adapted *Aerobacter cloacae.* The product 2-keto-D-gluconate-6-phosphate was isolated as a barium salt and methods of chromatographic separation and properties of this new intermediate are described. It is metabolized by crude extracts from *Aerobacter cloacae*, resulting in the formation of 3-phosphoglycerate and probably 6-phosphogluconate (54).

Considerable progress has been made in the elucidation of the steps of the Entner-Doudoroff pathway (55, 56). The enzyme which catalyzes the dehydration of 6-phosphogluconate (6-PG dehydrase) has been purified twenty-seven-fold from *Pseudomonas fluorescens* and separated from 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPG aldolase). Ferrous ions

(4×10^{-3} *M*) and glutathione (10^{-2} *M*) are required for maximal activity, partial replacement for Fe^{++} ions by Mn^{++} or Mg^{++}, for glutathione by cysteine or thiolglycolate was observed.

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IV. THE CITRIC ACID CYCLE

The major developments with respect to the citric acid cycle during the past year can be summarized as occurring along the following lines: (*a*) recognition that alternate reactions may exist within the framework of the cycle, (*b*) further evidence that the cycle exists in organisms that had previously been reported not to possess the cycle, (*c*) studies on the intimate mechanisms of action of the various enzymes of the cycle.

General Aspects of the Krebs Citric Acid Cycle

The Krebs cycle in microorganisms.—It has become increasingly clear that evidence for the existence of the citric acid cycle cannot be taken solely from experiments on whole cells. For example, Saz & Krampitz (1) have found that preparations of *Micrococcus lysodeikticus* do not oxidize citrate at an appreciable rate. However, extracts of these cells rapidly oxidize citrate to α-ketoglutarate. It is also of interest to note that although succinate oxidation by whole *M. lysodeikticus* cells are not inhibited by malonate, cell free extracts are strongly inhibited. The above experiments certainly demonstrate that the cell permeability for di- and tricarboxylic acids must be

considered when attempting to establish the citric acid cycle in intact cells.

Citric acid cycle members apparently vary in their capacity to penetrate into different microorganisms. Although whole cell suspensions of *M. lysodeikticus* do not oxidize citrate, intact *Pasteurella tularensis* cells will readily oxidize this tricarboxylic acid (2). The literature of the past has indicated that while a large number of microorganisms will handle citric acid cycle members, many others will not. It is therefore essential that to definitely rule out the Krebs cycle, conclusive experiments on cell free extracts must be carried out. In this connection it is of interest that evidence has recently been obtained which strongly suggests the existence of the cycle as a major respiratory pathway in *Escherichia coli* (3, 4, 5).

Strauss (6) has obtained data indicating that acetate is oxidized via the tricarboxylic acid cycle in *Neurospora*; however, this organism may also oxidize acetate by a dicarboxylic cycle under certain conditions. It is of interest in this connection that Ajl & Wang (3) have been unable to find any evidence for an acetate to acetate condensing enzyme in extracts of *E. coli*. These authors feel that the dicarboxylic cycle as previously suggested, as a mechanism for the oxidation of acetate in resting cells of this bacterium may be doubtful.

Englesberg & Levy (7) have shown that a strain of *Pasteurella pestis* when adapted to an oxidative metabolism (i.e., the oxidation of pyruvate, acetate, the C-4 dicarboxylic acids, etc.) demonstrates a specific induced synthesis of the citric acid cycle enzymes and also of phosphotransacetylase. As a result of this aerobic adaptation, there is a decrease in the levels of phosphofructokinase and triosephosphate dehydrogenase. This strain of *P. pestis* would appear to be an excellent example to illustrate that the concentration of both glycolytic and oxidative enzymes are subject to conditions under which the organism is grown.

Factors influencing the tricarboxylic acid cycle in animal tissues.—Katz & Chaikoff (8) have studied the metabolism of C^{14} labelled acetate in liver slices of fed and fasted rats. Fasting induced a marked decrease in the incorporation of C^{14} labelled acetate into the di- and tricarboxylic acids of the liver slices. The carboxyl carbon of acetate was oxidized to CO_2 at three times the rate of the methyl carbon; the methyl carbon, however, was found to be incorporated into glucose at three times the rate of carboxyl carbon. The methyl carbon was also incorporated into glutamate at a faster rate.

Foster & Villee (9) have studied pyruvate and acetate oxidation in the isolated rat diaphragm. It was found that the oxidation of acetate-1-C^{14} to CO_2 by the diabetic diaphragm was less than by the control tissue; addition of insulin was without influence on the oxidation of acetate by the diabetic tissue. Suggesting that CO_2 fixation is a major phenomenon of diaphragm muscle, Foster & Villee also report that the carboxyl carbon of pyruvate is incorporated in appreciable amounts in di- and tricarboxylic acids. This incorporation is somewhat depressed in the diabetic diaphragm. The authors also suggest that the lowered pyruvate oxidation may be related to a block, as a result of insulin deficiency, in the utilization of acetyl coenzyme A by the

condensing enzyme. In discussing such a view, the authors refer to experiments in which presoaking of diaphragm slices results in a lowering of the rate of pyruvate utilization which is comparable to the rate of diabetic slices. Presoaking has no effect on the diabetic tissue. Foster & Villee suggest the possibility that in the diabetic tissue there is a cofactor loss which may be reproduced in normal tissues by presoaking. Hence the decreased pyruvate oxidation in the diabetic diaphragm may be a manifestation of the loss of a factor rather than a direct lack of insulin.

Nearly all of the counts of pyruvate incorporated by the normal diaphragm slices can be accounted for, but only 60 to 75 per cent of the counts could be accounted for in the diabetic tissue. Acetate is utilized by the diabetic tissue at a somewhat faster rate than by the normal diaphragm tissue. However, only a small per cent of the acetate counts could be accounted for in the diabetic system. Foster & Villee suggest the possibility that the missing counts are present in the ketone substances. This would be of interest since synthesis of ketone substances has generally been considered essentially a function of liver.

Enzymes of the Tricarboxylic Acid Cycle

Pyruvate oxidase system.—The mechanisms involved in pyruvate oxidation have been reviewed recently by Gunsalus, Horecker & Wood (10). Although the general pattern of the pyruvate oxidase system has been clarified somewhat, much information is still required on the mechanisms involved in different animal tissues and also in various microorganisms. This is particularly the case in those systems in which the requirement of lipoic acid, pyridine nucleotides, and coenzyme A has not been clearly demonstrated.

Seaman & Naschke (11) report that lipoic acid can be removed from extracts of *Tetrahymena pyriformis* and of *Streptococcus faecalis* by adsorption on alumina. The pyruvate oxidases of these preparations are lost by such treatment and can be restored by the addition of lipoic acid. However, the reduction of ferricyanide when pyruvate is the electron donor is not influenced by the above alumina treatment of the *T. pyriformis* system (12). This is in contrast to the finding of Dolin (13) that diacetyl oxidation in *S. faecalis* using ferricyanide as electron acceptor requires lipoic acid. It is possible that in the *Tetrahymena* system (12), lipoic acid is still essential for reduction of ferricyanide since the alumina only removes approximately 50 per cent of the bound lipoic acid. Further work appears to be essential before the role of lipoic acid as a mediator of electron transfer in ferricyanide reduction can be definitely resolved.

Wolfe & O'Kane (14) have restudied the mechanism by which *Clostridium butyricum* extracts fix CO_2 into pyruvate. They report that this reaction requires as cofactors, cocarboxylase, CoA, and either phosphate or arsenate. These factors are the same as that required for the phosphoroclastic cleavage of pyruvate into acetyl phosphate, H_2, and CO_2. In addition the phosphoroclastic split also requires ferrous ions. It is of interest to note that although iodoacetate is a strong inhibitor of the phosphoroclastic reaction, it has little

effect on the fixation of CO_2 into pyruvate. The CO_2 fixing system is also much more stable than the phosphoroclastic system. It is suggested by the authors that the fixation reaction occurs early in the sequence of reactions involved in the phosphoroclastic split of pyruvate. However, as the authors also point out, the large requirement of cofactors for CO_2 fixation makes it most difficult to develop a working hypothesis at the present time. The complexity of the phosphoroclastic reaction is further indicated by the fact that labelled acetyl phosphate is not reincorporated into pyruvate by *C. butyricium* extracts. The same extracts, however, which do not promote acetyl phosphate incorporation will still promote a slow exchange of acetate into pyruvate. The phosphoroclastic split of pyruvate by *C. butyricum* seems to be a most complex system; this complexity is also indicated in the phosphoroclastic split of pyruvate to acetyl phosphate and formate (15).

Oxidation and activation of acetate.—Aisenberg & Potter (16) report that concentrations of sodium fluoride as low as 1×10^{-4} *M* will inhibit the activation of acetate; the activation of acetate was measured by CO_2 liberation from labelled acetate and the incorporation of the labelled acetate into non-volatile substances. Release of CO_2 from pyruvate C^{14} is not affected by this concentration of fluoride. It is suggested by these authors that fluoride inhibits the formation of acetyl CoA from acetate. Dinitrophenol was also found to depress acetate activation and stimulate pyruvate oxidation. The dinitrophenol effect on acetate activation appears to be attributable to its effect of lowering ATP synthesis.

The mechanism by which acetate is activated (that is, the formation of acetyl CoA) has recently been clarified by Berg (17), who has obtained evidence that the synthesis of acetyl coenzyme A proceeds by the following two reactions:

$$\text{ATP} + \text{acetate} \rightleftharpoons \text{adenyl acetate} + \text{pyrophosphate} \qquad 1.$$

$$\text{Adenyl acetate} + \text{CoA} \rightleftharpoons \text{acetyl CoA} + \text{adenylic acid} \qquad 2.$$

$$\text{Net: ATP} + \text{acetate} + \text{CoA} \rightleftharpoons \text{pyrophosphate} + \text{adenylic acid} + \text{acetyl CoA}$$

Adenyl acetate has been synthesized and shown to react with pyrophosphate to form ATP, and with CoA to form acetyl CoA. It seems, therefore, that the primary mechanism in the activation of acetate involves an acyl cleavage of ATP resulting in the liberation of inorganic pyrophosphate and the generation of adenyl acetate. The above formulation is supported by the O^{18} exchange experiments of Boyer *et al.* (18) which indicate that an acyl cleavage of ATP occurs in the activation of carboxyl groups.

It is of importance that, as previously reported by Jones *et al.* (19), the pyrophosphate exchange reaction with ATP for the activation of acetate does not involve CoA. The findings of Berg would also be in accordance with the observations of Jencks (20), that coenzyme A is not essential for the liberation of pyrophosphate from ATP in the presence of octanoic acid.

Although adenyl acetate can be converted to either ATP or acetyl coenzyme A, no evidence has as yet been obtained for the enzymatic synthesis of the compound from acetyl CoA or ATP and acetate (see reactions 1 and 2).

Final proof for the postulated mechanism, therefore, must await experiments on the enzymatic formation of adenyl acetate.

Brady (21) and Elliott & Marcus (22) have described the preparation and some of the properties of fluoroacetyl CoA. The compound is active in the phosphotransacetylase, thiotransacetylase, and arylamine acetylase systems. In the presence of the condensing enzyme and oxalacetate, fluoroacetyl CoA is converted to fluorocitrate. Fluoroacetyl coenzyme A is a potent competitive inhibitor of acetyl coenzyme A in the condensing reaction.

Partially purified acetate activating systems from yeast and liver do not form fluoroacetyl CoA from fluoroacetate, ATP, and CoA (21). This is of interest, since fluoroacetate toxicity appears to be a result of the formation of fluorocitrate which inhibits aconitase activity; hence in order for fluoroacetate to be toxic, it must be first activated to fluoroacetyl CoA. However, Brady reports that an enzyme from rabbit kidney will activate fluoroacetate, as measured by hydroxamic acid formation. This activation of fluoroacetate appears to be dependent on CoA. It is possible that the formation of fluoroacetyl CoA occurs by a coenzyme A transphorase type of reaction, from acetyl CoA according to the following equation:

$$\text{Acetyl CoA} + \text{fluoroacetate} \rightarrow \text{fluoroacetyl CoA} + \text{acetate} \qquad 3.$$

Brady & Stadtman (23) have studied the transfer of the acetyl group from acetyl CoA to other thio compounds. These reactions are referred to as thiotransacetylases, and three distinct enzymes of this type have been found in pigeon liver preparations. Thiotransacetylase A catalyzes a transfer between acetyl CoA and reduced lipoic acid to form acetyl lipoic acid. The enzyme designated as thiotransacetylase B promotes the acetylation of 2 mercaptoethylamine by acetyl CoA but not of lipoic acid. The third type of enzyme promotes the acetylation of H_2S to form thioacetic acid. Similar thiotransacetylating enzymes have been found in crude extracts of *Clostridium kluyveri* as well as in pigeon liver. It is of interest that brain contains thiotransacetylases which do not act on acetyl CoA (24).

Acetate cannot only be activated by ATP to form adenyl acetate, but also can be activated to form acetyl phosphate. The enzyme which catalyzes this transfer of phosphate from ATP to acetate to form acetyl phosphate and ADP has been termed acetokinase. The reaction is somewhat reversible and is given in the following equation:

$$\text{ATP} + \text{acetate} \rightleftharpoons \text{ADP} + \text{acetyl phosphate} \qquad 4.$$

The equilibrium of the reaction as determined by Rose *et al.* (25) is largely in favor of the formation of ATP; the ΔF toward ATP synthesis is -2800 calories. The acetokinase from *E. coli* has been extensively purified and acts specifically on acetate. Other fatty acids do not react at any significant rate, and hence the enzyme can be used as a means of quantitatively determining acetate. This is achieved by measuring the acetyl phosphate formed from ATP and acetate as the hydroxamic acid. The mechanism of the acetokinase reaction has been suggested by Rose *et al.* (25) to be a phosphate transfer reaction, involving a single displacement. This mechanism would be the same

as that described for pyruvic acid kinase (26) and for the phosphoglyceric acid kinase (27).

Aconitase.—Morrison (28) has reported that dialysis of aconitase results in a great loss of activity. Preincubation of the dialyzed preparation for 1 hr. with ferrous ions produces a fifteen-fold increase in activity. Cysteine, glutathione, ascorbic acid, and thioglycolic acid have little effect on the dialyzed aconitase; however, when any one of these compounds is added with ferrous ions, a 70-fold increase in activity is observed. From Morrison's data it appears that 4 moles of glutathione per atom of iron are required on the active site of the enzyme.

Aconitase activity is decreased in the liver mitochondria of scorbutic animals as reported by Takeda & Hara (29). Addition of ferrous ions or of ferrous ions plus ascorbic acid partially restored the lowered activity. It is suggested that the primary lesion in ascorbic acid deficiency is a mobilization of ferrous ions; this results in the inactivation of aconitase, since this enzyme requires ferrous ion.

Both synthetic and enzymatically prepared fluorocitrates have been found to be competitive inhibitors of the purified aconitase reaction (30). However, no clear cut irreversible inhibition of the aconitase by the fluorocitrate has been demonstrated. The synthetic compound has a smaller K_m for inhibition than the natural compound; this is reversed in kidney particles where the natural fluorocitrate is a more potent inhibitor. The nature of the fluorocitrate isomer which causes inhibition is still unresolved. It is of interest to note that fluorocitrate does not inhibit isocitric dehydrogenase.

Speyer (31) in preliminary studies with D_2O has obtained evidence that citrate may be converted to isocitrate by aconitase without the intermediate formation of aconitate. This author has postulated that a common carbonium ion-ferrous ion-enzyme complex is formed from all three tricarboxylic acids. This complex may add hydroxyl to form either citrate or isocitrate, and by loss of a proton yield *cis*-aconitate.

The prevalent opinion has been that the conversion of aconitate to either isocitrate or citrate is the function of a single protein (32, 33). Neilson (34), however, reports that by fractionation of *Aspergillus niger* extracts the two activities can be separated and thus concludes that in this organism the aconitase consists of at least two enzymes. That Neilson's results may differ from earlier studies because of the source of the aconitase remains a point to be clarified.

Isocitric dehydrogenase.—Crude cell free extracts of *A. niger* have been found to contain both DPN and TPN isocitric dehydrogenases (35). The DPN system requires 5′-adenylic acid and Mg^{++} for activity. The TPN enzyme requires only Mg^{++} (or Mn^{++}). These observations are similar to those which describe yeast (36) and animal tissue (37) as containing two isocitric dehydrogenases.

2′-Adenylic acid has been found to inhibit TPN isocitric dehydrogenases but not the DPN enzymes (38). This observation has been used to dis-

tinguish in crude extracts whether the TPN and DPN catalyzed reactions are attributable to one or two proteins.

The occurrence of separate DPN and TPN linked isocitrate dehydrogenases appears to be of interest in connection with factors that regulate the citric acid cycle. Villee & Gordon (39, 40, 41) have found a stimulation by estradiol of the DPN isocitric dehydrogenase from human placenta homogenates. The hormone does not act on the placenta TPN enzyme. The DPN is present primarily in the supernatant (nonparticulate fraction), whereas the TPN dehydrogenase is largely in the mitochondria. Villee & Gordon (41) suggest the possibility that estradiol is involved as a mediator in the transfer of hydrogen from isocitrate to DPN, and in this process it is reversibly converted to estrone.

Ramakrishnan *et al.* (42) report that during citric acid accumulation in *A. niger* the concentration of condensing enzyme increases whereas the isocitric dehydrogenase and aconitase activities are lost. Accumulated citric acid has been found to inhibit the isocitric dehydrogenase of *A. niger*. The mechanism of this inhibition is not clear.

Succinic dehydrogenase.—The succinic dehydrogenase from beef heart has been solubilized and purified by Singer & Kearney (43). These workers have found that the purified enzyme is a flavoprotein containing iron (44, 45, 46). The flavin component does not appear to be flavin adenine dinucleotide but is a riboflavin derivative containing 5′-adenylic acid (46).

Evidence has been obtained by Bueding *et al.* (47) that DPN and FAD are components of the succinoxidase complex of the parasitic worm *Ascaris lumbricoides*. This is the first indication that pyridine nucleotides are concerned in the succinate system. The finding that FAD is also a requirement for the succinoxidase reaction is interesting, particularly in view of the findings of Singer & Kearney (see above) that a flavin derivative is a component of the extensively purified succinic dehydrogenase. In this connection, it is also of interest that Bril (48) has reported that in rat liver homogenates, DPN and other factors are involved in the reduction of triphenyltetrazolium chloride when succinate is the electron donor.

Repaske (49) reports the apparent solubilization of the succinic dehydrogenase of *Azotobacter vinelandii*. Some properties of the enzyme have been described. The succinic dehydrogenase from *Rhodospirillum rubrum* has also been partially pyrified (50).

It has been found that under several experimental conditions in which oxalacetate accumulates, succinate oxidation is inhibited (51). The dehydrogenation of succinate using formazan as electron acceptor is also decreased as the result of oxalacetate accumulation (52). Hence, it appears that the rates of formation and disappearance of oxalacetate may be of significance in regulating the rate of the tricarboxylic acid cycle, because of the inhibition of the succinate system by the beta keto acid.

Englard & Colowick (53) have studied the mechanism of the anaerobic exchange of succinate with D_2O, an exchange which is catalyzed by succinic

dehydrogenase preparations. In this exchange reaction deuterium becomes incorporated in an unspecific manner into the succinate, since oxidation of the labelled succinate results in the formation of labelled fumarate. This suggests that the succinic dehydrogenase reaction is not stereospecific and that a direct hydrogen transfer is not involved in the oxidation of succinate.

Fumarase.—Fisher *et al.* (54) have studied the mechanism of the fumarase reaction. They have found that the enzymatic hydration of fumarate to malate is stereospecific, this conclusion having been reached after carrying out experiments in D_2O. It is clear from these studies that the hydrogen atom is placed exclusively in one of the two possible positions in the hydration reaction and removed from that position in the dehydration reaction. Fisher *et al.* have also found that the incorporation of D into malate in the presence of D_2O is faster than the conversion of malate to fumarate. This has been taken as evidence for an intermediate whose nature is that of malate with one methylene hydrogen removed. Frieden & Alberty (55) have studied in some detail the kinetics of the fumarase reaction and have developed from their data a simple mechanism which allows for the ionization of the enzyme and for the enzyme-substrate complexes.

Malic dehydrogenase.—The malic dehydrogenase from wheat germ has been found by Loewus *et al.* (56) to promote a direct transfer of hydrogen from substrate to DPN. The stereospecificity of the malic dehydrogenase reaction with respect to DPN is the same as that of yeast alcohol dehydrogenase. The reduction of oxalacetic acid in D_2O indicates that the keto form of the acid is reduced and that the enol form is not involved. Similar observations have been made with pyruvate and acetaldehyde in lactic and alcohol dehydrogenases.

Fixation of CO_2 into dicarboxylic acids.—Several distinct reactions have been described for the fixation of CO_2 into either malic or oxaloacetic acid. These are summarized in the following equations:

$$\text{Pyruvate} + CO_2 + \text{TPNH} \rightarrow \text{TPN} + \text{malate} \qquad 5a.$$

$$\text{Phosphoenol pyruvate} + CO_2 + \text{IDP} \rightarrow \text{ITP} + \text{oxalacetate} \qquad 5b.$$

$$\text{Phosphoenol pyruvate} + CO_2 \rightarrow \text{P} + \text{oxalacetate} \qquad 5c.$$

Reaction 5a is catalyzed by the "malic" enzyme which has previously been studied in great detail by Ochoa and co-workers (57). Reaction 5b is the system in animal tissues recently elaborated by Utter and associates (58, 59, 60) and represents a truly reversible reaction.[4] Reaction 5c is the phosphoenolpyruvate carboxylase recently purified by Bandurski (61) from spinach leaf tissue. This reaction is not reversible and does not involve nucleotides. Nor does the phosphoenolpyruvate carboxylase catalyze any appreciable exchange between $C^{14}O_2$ and the carboxyl group of oxaloacetate in the

[4] Reaction 5b is written with IDP since the hypoxanthine nucleotides are more effective than the adenine nucleotides in animal tissues. However, the possibility exists that the adenine nucleotides may react more favorably in nonanimal systems (62).

presence or absence of polynucleotide or inorganic phosphate. This exchange reaction is a distinct characteristic of the Utter system (reaction 5b).

Tchen & Vennesland (62) report two distinct systems in wheat germ extracts, catalyzing reactions 5b and 5c. These appear to be separate enzymes. Since reaction 5c requires only very low CO_2 tensions, Tchen & Vennesland suggest that this reaction may act as a trap for CO_2 in the dark. The trapping in the dark of CO_2 (arising from respiration) may then be a factor in explaining the accumulation of malic acid in the dark. It is of interest to note that much higher concentrations of CO_2 are required for reactions 5a and 5b than for reaction 5c.

The mechanism of reaction 5c has been studied by Tchen *et al.* (63) by carrying out the reaction in D_2O. No evidence was obtained that the enol form of oxalacetate participated in the reaction. The results also permit the conclusion that if a phosphorylated derivative is an intermediate, the phosphate must be attached to a carboxyl group. Despite the large amount of excellent research carried out on reactions 5a, 5b, and 5c, the intimate mechanisms involved in these systems are far from resolved.

Metabolism of propionate.—It has previously been reported that propionate may be metabolized to lactate with the possible intermediate formation of acrylate (64). Flavin, Ortiz & Ochoa (65) have reported from experiments with animal tissues a mechanism for propionate oxidation which does not involve the formation of lactate. These authors report evidence that CO_2 fixation and the intermediate formation of succinate are involved in propionate oxidation. The scheme of Flavin *et al.* is summarized below:

$$\text{ATP} + \text{CoA} + \text{propionate} \rightarrow \text{propionyl CoA} \qquad \text{6a.}$$

$$\text{Propionyl CoA} + CO_2 \rightarrow \text{methyl malonyl CoA} \qquad \text{6b.}$$

$$\text{Methyl malonyl CoA} \rightarrow \text{succinyl CoA} \qquad \text{6c.}$$

$$\text{Succinyl CoA} + H_2O \rightarrow \text{succinate} \qquad \text{6d.}$$

$$\text{Succinate} + O_2 \rightarrow CO_2 + H_2O \qquad \text{6e.}$$

Reaction 6c is a postulated reaction, and there is no evidence as yet for this type of rearrangement. Reaction 6e simply indicates that the succinate formed from the propionate is oxidized through the citric acid cycle. The above scheme is of considerable significance since it indicates that succinyl CoA can be formed from reactions other than α-ketoglutarate oxidation. It is also of importance in demonstrating that there are diverse mechanisms through which substances enter the tricarboxylic acid cycle.

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IV. The Citric Acid Cycle

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V. VARIATIONS ON THE CITRIC ACID CYCLE

At various times in the past, metabolic cycles have been proposed which differ from the now classical citric acid cycle in that they involve either C_5 acids or C_7 acids instead of the C_6 acid, citrate.

Although there is still no evidence for the operation of such cycles, the discovery during recent years of new acids which are structurally similar to known citric acid cycle intermediates has aroused new interest in the possibility of the existence of "homologous" citric acid cycles. Several hypothetical variations will be considered in the following discussion.

Itaconic acid metabolism.—Although it has been known to occur in nature since 1929, when it was first isolated from an aspergillus by Kinoshita (1), itaconic acid has until very recently received little attention from biochemists. Indeed, almost all the previous work has dealt with attempts to improve the microbial production of itaconic acid since it is a compound with a large potential industrial market.

During 1955, several papers appeared which are concerned with the mechanism of itaconate biosynthesis. Working with the fungus, *Aspergillus*

terreus, Eimhjellen & Larsen (2) have shown that the products of glucose metabolism were dependent upon the pH at which the organism was grown. At pH 6 the products of glucose oxidation were those expected from the functioning of a classical citric acid cycle, i.e., malate, succinate, fumarate, and CO_2. In contrast, at pH 2 the products were almost exclusively itaconate and CO_2. The conclusion was reached that the system converting glucose to itaconate is formed only at acid pH.

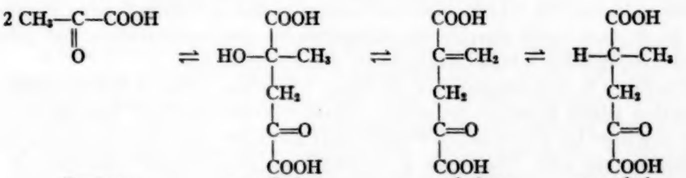
In the second paper of the series (3) a study was made of the effect of various substrates and inhibitors on itaconate production. The substrates tested fell into three classes: (*a*) substrates causing a definite increase in itaconate production: glucose, maltose, glycerol, ethanol, glycerate, citrate, malate; (*b*) substrates which neither increase nor decrease itaconate formation: *cis*-aconitate, citramalate; and (*c*) substrates which inhibit the endogeneous synthesis of itaconate: pyruvate, acetate, oxalacetate, and glycollate. Fluoroacetate, at $2\times10^{-4}M$ increased the molar conversion of both glucose and citrate to itaconate. Arsenite, at $1\times10^{-2}M$, inhibited the conversion of glucose to itaconate and led to the accumulation of pyruvate and acetaldehyde. The authors conclude that glucose is converted to itaconate via an activated citrate and that pyruvate is an intermediate in this conversion. That itaconate can be formed from citrate has already been demonstrated by Corzo & Tatum (4) who found that the conversion involved the loss of the carbon atom derived from the acetate carboxyl group and that the carbon atom derived from the methyl group of acetate becomes the methylene carbon atom of itaconate. The demonstration by Bentley & Thiessen (5) of an enzyme in this same organism which can decarboxylate *cis*-aconitate to itaconate and CO_2 is consistent with this idea. It need hardly be emphasized, however, that the mere presence of this enzyme in the organism does not prove that itaconate is formed *in vivo* by a direct decarboxylation of *cis*-aconitate. As mentioned previously, *cis*-aconitate had no effect on the endogeneous itaconate production in Larsen & Eimhjellen's experiments, although in the presence of glucose plus *cis*-aconitate, there was a stimulation in itaconate formation (3). A consideration of other possible routes for itaconate synthesis will be postponed until the next section where the synthesis of very similar compounds will be discussed.

Naturally occurring acids which are structurally related to citric acid cycle intermediates.—During 1955 many reports have appeared which describe the isolation of new carboxylic acids which bear a structural resemblance to known citric acid cycle intermediates. Using predominantly techniques of paper chromatography, workers in several different laboratories have examined various plant tissues for the occurrence of new acids. The search has been a rewarding one.

In 1951 and 1952 Done & Fowden described the isolation of two new amino acids, γ-methylene glutamate and γ-methylene glutamine from groundnut plants (6, 7). More recently, Steward and his group in this country have isolated these compounds from tulips (8). A transaminase system has

been demonstrated in groundnut plants which can convert γ-methylene glutamate to γ-methylene-α-ketoglutarate (9). On the strength of this conversion, an attempt was made to demonstrate the natural occurrence of the latter compound in this plant. This has now been accomplished by Fowden & Webb (10). Towers & Steward had previously reported the occurrence of γ-methylene-α-ketoglutarate in tulips (11), but there is an apparent discrepancy between the melting points of the 2,4 dinitrophenylhydrazone derivative obtained by Towers & Steward and that found by the English group.

Two compounds which are closely related structurally to γ-methylene-glutamate have also been isolated from plant material. γ-Methylglutamate and γ-methyl-γ-hydroxyglutamate, the reduced and hydrated analogues respectively of the unsaturated compound, have been demonstrated in a fern by Virtanen & Berg (12). Independently, γ-methyl-γ-hydroxyglutamate has been found by Steward and his group, in addition to the corresponding keto acid, γ-methyl-γ-hydroxy-α-ketoglutarate (13).



Pyruvate — γ-methyl-γ-hydroxy-α-ketoglutarate — γ-methylene-α-ketoglutarate — γ-methyl-α-ketoglutarate

FIG. 1. Interrelations of various ketoglutarate derivatives and a possible mode for their biosynthesis.

There is no evidence which would indicate how this series of compounds is synthesized *in vivo*. Steward *et al.* (13) have suggested that γ-methyl-γ-hydroxy-α-ketoglutarate could be synthesized by a condensation of two pyruvates, a reaction which is used in the chemical preparation of the compound. The reaction would be analogous to that catalyzed by the condensing enzyme of Ochoa (14) and would probably require activation of at least one of the substrates. γ-Methylene-α-ketoglutarate could then be formed from the hydroxy compound in a fumarase-like reaction and the γ-methylderivative could be formed in a subsequent reductive step. This hypothetical scheme is outlined in Figure 1.

It is interesting to note that the product of oxidative decarboxylation of γ-methylene-α-ketoglutarate would be itaconate, thus providing an alternate possibility for the synthesis of this compound. Several variations of this type of synthesis are possible. The analogous condensation between acetyl CoA and pyruvate would yield citramalate (α-hydroxy, α-methyl succinate) which on loss of water could give itaconate. It is suggestive that citramalate has recently been isolated from plant tissue (15). Another possible metabolic

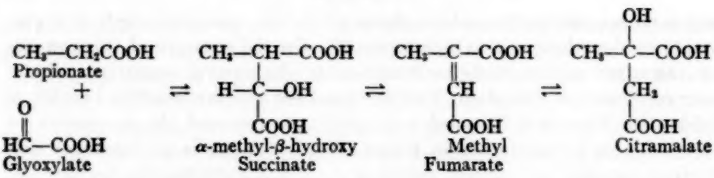


FIG. 2. Scheme for the possible synthesis of citramalate.

route for itaconate synthesis via citramalate would involve the preliminary formation of the isomeric compound, α-methyl-β-hydroxysuccinate. This scheme is shown in Figure 2. The condensation of propionate (as the CoA derivative) and glyoxylate would give α-methyl-β-hydroxy succinate which by a series of reactions analogous to the aconitase-catalyzed conversion of citrate to isocitrate would give citramalate. The recently discovered isocitritase (16, 17, 18), which catalyzes the conversion of isocitrate to succinate and glyoxylate, could provide the substrates for this condensation, the propionate being formed from succinate by decarboxylation.

Bentley (5) has suggested a pathway for γ-methylene-α-ketoglutarate formation which involves the seven carbon tricarboxylic acid first isolated from dog liver by Rapoport & Wagner (19) and later shown to be formed in *Escherichia coli* (20). The types of chemical reactions required for this conversion are outlined in Figure 3, although there are many variations possible in the sequence of the reactions.

There would seem to be no evidence at the present time which would in-

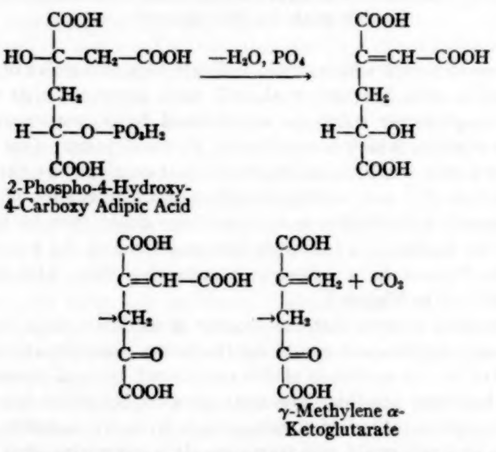


FIG. 3. Proposed scheme for synthesis of γ-methylene-α-ketoglutarate from Rapoport's compound.

dicate which of the many possible schemes for the synthesis of these unsaturated acids is most likely. Some of them, such as the proposed condensation between acetyl CoA and pyruvate to form citramalate which could be dehydrated to form itaconate, are not consistent with the isotopic findings of Corzo & Tatum (4). A final decision will obviously have to wait until more data are available. Details of the isotope experiments would seem to be particularly desirable.

Virtanen and his group have reported the isolation of several other new acids from plants. γ-Hydroxyglutamate has been found in *Phlox* (21) and both β-hydroxy-α-ketobutyrate and γ-hydroxy-α-ketobutyrate have been isolated from cowberries (22). The last two compounds are the keto-analogues of threonine and homoserine respectively. The enzymatic synthesis of γ-hydroxy-α-ketobutyrate from pyruvate and formaldehyde has already been described with an enzyme isolated from beef liver (23). The analogous condensation between pyruvate and glyoxylate would yield γ-hydroxy-α-ketoglutarate which could, on transamination (24), yield γ-hydroxyglutamate.

Three-point attachment.—Recently, Schwartz & Carter (25) have proposed that a compound having one carbon which bears two identical groups and two dissimilar groups (meso carbon) is capable of asymmetric synthesis when it is reacted with an optically active reagent by virtue of the nonsuperimposability of the two halves of the meso carbon groupings. Wolfrom (26) has pointed out that the effect may depend upon the reverse reaction in which energetically different diastereoisomers react at different rates to set up an equilibrium in which the products are present in unequal concentrations. In either case, in view of the complex and, presumably, wholly specific structure of enzymes, the question may be raised whether three points of attachment are necessary to account for the asymmetric synthesis and cleavage of symmetrical molecules, such as citric acid, or whether the meso carbon hypothesis can account for these reactions. A case in point may be the observation of Bublitz & Kennedy (27) that asymmetrically labeled glycerol, prepared by the fermentation of glucose labeled with C^{14} in the 3 and 4 positions, is phosphorylated by a purified glycerokinase to yield glycerophosphate in which 83 per cent of the radioactivity is in the carbon atom bearing an unphosphorylated primary hydroxyl group. Since the C^{14} in chemically synthesized glycerol, and in the L-α-glycerophosphate derived from it, is equally distributed between phosphorylated and unphosphorylated hydroxyl groups, isotope effects seem not to be involved. While the Ogston hypothesis (28) would seem to require absolute specificity in a reaction of this type, the observed results do not seem to be inconsistent with the mesocarbon concept.

V. Variations on the Citric Acid Cycle

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VI. PHOTOSYNTHESIS

The fixation of carbon dioxide.—The formation of carbohydrate from CO_2 has been ascribed (1 to 4) to the following series of reactions:

$$\text{6 ribulose diphosphate (RuDP)} + 6\,CO_2 \rightarrow \text{12 phosphoglycerate (PGA)} \qquad 1.$$

$$\text{12PGA} + \text{12 ATP} + \text{12 DPNH} \rightleftharpoons \text{12 triose phosphate} + 12\,P_i + \text{12 DPN}^+ + \text{12 ADP} \qquad 2.$$

$$\text{10 triose phosphate} \xrightleftharpoons{\text{aldolase}} \text{5 fructose diphosphate (FDP)} \qquad 3.$$

$$\text{5 FDP} \xrightleftharpoons{\text{fructose diphosphatase}} \text{5 fructose 6-phosphate (F-6-P)} + 5\,P_i \qquad 4.$$

$$\text{2 F-6-P} + \text{2 triose phosphate} \xrightleftharpoons{\text{transketolase}} \text{2 pentose phosphate} + \text{2 tetrose phosphate} \qquad 5.$$

$$\text{2 F-6-P} + \text{2 tetrose phosphate} \xrightleftharpoons{\text{transaldolase}} \text{2 sedoheptulose phosphate (S-7-P)} + \text{2 triose phosphate} \qquad 6.$$

$$\text{2 S-7-P} + \text{2 triose phosphate} \xrightleftharpoons{\text{transketolase}} \text{4 pentose phosphate} \qquad 7.$$

$$\text{6 pentose phosphate} + \text{6 ATP} \xrightarrow{\text{phosphoribulokinase}} \text{6 RuDP} + \text{6 ADP} \qquad 8.$$

$$\text{Sum: } 6\,CO_2 + \text{18 ATP} + \text{12 DPNH} \rightarrow \text{F-6-P} + 17\,P_i + \text{18 ADP} + \text{12 DPN}^+ \qquad 9.$$

The key role of phosphoglycerate as the primary product of photosynthesis has been confirmed by fixation experiments of 0.1 sec. duration, in which this was the only compound to become labeled (5). The enzyme which catalyzes the formation of PGA (reaction 1) has been referred to as carboxydismutase, RuDP carboxylase, and carboxylation enzyme. It has been purified from spinach leaves and from *Chlorella* extracts and shown to catalyze the formation of two moles of PGA from RuDP and CO_2 (3, 4, 6). The substrate, RuDP, has been prepared from ribose 5-phosphate by the combined action of phosphoriboisomerase and phosphoribulokinase (reaction 8), and evidence obtained for esterification in the 1-position (7).

PGA formed in the carboxylation reaction may be reduced and converted to fructose monophosphate by the reversal of the glycolytic steps (reactions 2 and 3). Three glyceraldehyde 3-phosphate dehydrogenases have now been detected in leaf tissue. The latest to be reported (8) is active with TPN but does not require phosphate or arsenate. This enzyme, like the previously described phosphate-TPN enzyme (9, 10), occurs only in green leaves and may therefore play a role in the photosynthetic process. However, the reduction of phosphoglycerate to triose phosphate (as in reaction 2) has not been shown to be catalyzed by this enzyme. Fructose diphosphatase (reaction 4) has been purified from spinach leaves (3).

The formation of pentose phosphate from hexose monophosphate may occur oxidatively by way of glucose 6-phosphate and 6-phosphogluconate, or by a series of nonoxidative rearrangements catalyzed by transketolase and transaldolase (reactions 5, 6, and 7). The available isotope data indicates that the second pathway predominates. This has been reviewed previously (1).

In the red alga, *Iridophycus fleccidum*, the final storage form of carbohydrate is α-D-galactosyl-2-glycerol, rather than starch or sucrose, but the intermediate products appear to be identical with those which occur in green plants (11). The final steps in the synthesis of galactosyl glycerol appear to involve a condensation of uridine diphosphate with α-glycerol phosphate.

The precursor-product relationship of RuDP and PGA has been studied in the intact cell (12) and evidence obtained for an alternate mechanism to that given in reactions 1 and 2. On the basis of quantitative measurements of changes in the concentration of these substances when CO_2 was removed, it was suggested that RuDP could undergo a reductive carboxylation to form one mole of PGA and one of triose phosphate.

Other CO_2 fixation mechanisms are present in plants, but most of these are not dependent on light, and their relation to photosynthesis is obscure. In *Rhodopseudomonas capsulatus*, Krebs cycle intermediates, serine, and other amino acids become labeled rapidly in the light or dark although the main mechanism for CO_2 fixation in the light is the phosphoglycerate cycle (2). In *Rhodopseudomonas gelatinosa* acetone is converted to acetate, with acetoacetate as the probable intermediate (13). This reaction requires either light or oxygen as a source of energy. Similarly, the incorporation of formate into glyceric acid and serine in barley leaves occurs only in the light (14).

The role of glycolic acid in plant metabolism also remains obscure. Roots of higher plants produce labeled glyoxylate and glycolate from $C^{14}O_2$ (15). These may possibly arise from labeled citrate or isocitrate by C_2-C_4 cleavage. In *Chlorella* large amounts of labeled glycolate are excreted during photosynthesis. In the absence of CO_2 this compound is not excreted but accumulates in the cells (12, 16).

Two other sugars formed in plant tissues may participate in photosynthesis. In avocado leaves mannoheptulose phosphate becomes labeled during brief photosynthesis in $C^{14}O_2$ (17); this ester is probably a precursor of the free sugar, mannoheptulose, which accumulates in the plant and which may be regarded as a storage carbohydrate. Erythrulose 1-phosphate is formed from C^{14}-formaldehyde and triose phosphate in Swiss chard leaf preparations (18). In these experiments, ribose also was labeled suggesting a relation of the tetrose ester to pentose metabolism and photosynthesis.

The energy requirement.—On the basis of the cyclic mechanism it has been estimated (3, 4) that 3 moles of ATP and 2 moles of DPNH or TPNH (4 equivalents of [H]) are required for the reduction of one molecule of CO_2 to carbohydrate (equation 9). Assuming 3 moles of ATP to be generated in the reoxidation of DPNH by 2[OH], then a total of 3 moles of DPNH (6 equivalents of [H]) are required. If one photon produces one equivalent each of [H] and [OH], it can thus be estimated that a minimum of 6 photons are required per mole of CO_2. New determinations of the quantum efficiency of photosynthesis in *Chlorella* yield average values of 7.4 and 8.9 (19, 20) with lower limits in some experiments approaching 6 to 7. At low levels of photosynthesis respiration may provide some or all of the ATP required and raise the quantum efficiency to values approaching 4. Recent measurements of the corrected quantum requirement as a function of the photosynthesis/respiration ratio (19) may resolve the difference between the two schools favoring quantum requirements of 8 or 4. However, there is some disagreement as to the validity of methods employed for the quantitative light measurements (21).

Additional evidence for the previously reported photochemical reduction of pyridine nucleotides has been obtained (22, 23), and phosphorylation with chloroplast preparations has been demonstrated (24 to 27). The spinach chloroplast preparations require FMN, Mg^{++}, and ascorbate for photosynthetic phosphorylation (27), and a stimulating effect of vitamin K derivatives has also been noted (28). In intact *Chlorella* in the absence of CO_2 polyphosphate accumulates (25). The availability of cell-free preparations which catalyze photosynthetic phosphorylation should greatly facilitate work on this mechanism. In particular, it will be of interest to establish the relation of the photooxidation of DPNH to this process (29).

Although the light reactions and CO_2 fixation are separable, a direct coupling of these processes in the intact cell is suggested by the fact that the entire process can be observed to occur in the isolated chloroplast (30, 31).

The function of chlorophyll.[5]—The transient existence of univalent

[5] The thoughts presented here were found in Dr. Korkes' notes and although not a "review" are presented on their own merit.

magnesium prepared by electrolysis with metallic magnesium electrodes provokes some speculation on the possible relationship of this phenomenon to photosynthesis. In essence, the chlorophyll molecule, for the purpose of this scheme, may be likened to any phosphor in which an impinging photon displaces an orbital electron to a metastable state in another locus in the complex. Since the hydration of chlorophyll increases with increasing alkalinity, the extrapolation that it is primarily hydroxyl ion which "solvates" seems to be permissible. Since phytins fail to show this dependence on pH, it can be assumed that this property depends on the presence of magnesium, and that the hydroxyl ion is bonded to the Mg atom. In a large number of crystalline magnesium compounds Mg appears to exhibit a coordination number of 6, which allows for the possible coordination of two hydroxyl ions per Mg in chlorophyll.

The following sequence may occur.

$$4\ \text{Chl-Mg}^{++} + 4\ \text{OH}^- \rightarrow 4\ \text{Chl-Mg}^{++} - \text{OH}^- \qquad 10.$$

$$4\ \text{Chl-Mg}^{++} - \text{OH}^- + 4\ h\nu \rightarrow 4\ \text{Chl-Mg}^+ + 4\ (\text{OH}) \qquad 11.$$

$$4\ (\text{OH}) \rightarrow \text{O}_2 + 2\ \text{H}_2\text{O} \qquad 12.$$

$$4\ \text{Chl-Mg}^+ + 4\ \text{H}^+ + 2\ \text{S-S} \rightarrow 4\ \text{Chl-Mg}^{++} + 4\ \text{SH} \qquad 13.$$

$$\text{Sum: } 4\ \text{OH}^- + 4\ \text{h} + 4\ \text{H}^+ + 2\ \text{S-S} \rightarrow \text{O}_2 + 4\ \text{SH} + 2\ \text{H}_2\text{O} \qquad 14.$$

In this sequence, four reducing groups (indicated as a lipoic acid derivative, for example) and one mole of oxygen are formed per four quanta.

The chlorophyll molecule serves two functions in this scheme; it chelates and imparts greater stability to univalent Mg than would be possible in the free state, and it provides the necessary long wavelength resonator to bring about the proposed electron displacement.

Fluorescence would be the reversal of reaction 11, and, since there are two products of this reaction, reversible bleaching of chlorophyll would be expected to be proportional to the square root of light intensity, which agrees with observation. The yield of fluorescence *in vivo* should be a function of oxidizable acceptor present, and previous observations on the increased fluorescence at high light intensities, which have been interpreted as depletion of a quenching acceptor, may alternatively be looked upon as the only consequence of light capture possible in the absence of acceptor. The total charge on the chlorophyll molecule is probably zero; the double positive charge is written on the Mg for emphasis of the thought that it is, in fact, the Mg atom which is responsible for the indicated valence change, while the rest of the molecule provides stability through resonance. Reaction 10 actually results in a complex of net charge -1; the extra electron derived from the hydroxyl group being responsible for this charge. This is shifted to a metastable state in the photochemical event, and finally transferred to a general (nonphotosynthetic) acceptor in reaction 13. These concepts may be reconciled with a large body of information already in the literature. To be sure, one might equally readily find incompatible observations, but they appear to warrant serious consideration as a working basis for future research.

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VII. SYNTHESIS OF SERINE FROM CARBOHYDRATE PRECURSORS

At least two pathways of serine biosynthesis from carbohydrate intermediates are evident from the literature of the past year. One involves serine formation from glycine derived from ribose-5-phosphate (1) or glycolic acid (2). In addition Ichihara & Greenberg (3) have observed conversion of phosphoglyceric acid to serine in the presence of alanine and DPN in a partially purified enzyme preparation from rat liver. Better yields of serine were ob-

tained from uniformly labeled glucose in the presence of sodium fluoride and a nitrogen atmosphere. Neither pyruvic or lactic acid gave rise to serine. The best yield of serine was obtained by transamination of 3-phosphohydroxypyruvic acid by alanine. Black, Kleiber & Baxter (4) found that alanine and serine had the highest specific activity of 14 amino acids isolated from the casein of a dairy cow after injection of uniformly labeled glucose. It is not known which of the intermediates in this serine synthesis are phosphorylated since reactions exist for both the phosphorylated and unphosphorylated compounds. A specific alanine-hydroxypyruvic transaminase was obtained by Sallach (5) from dog liver acetone powder. This may be similar to the system outlined by Meister, Fraser & Tice (6) who found that hydroxypyruvic acid could be transaminated by glutamine to serine in a partially purified extract of rat liver acetone powder. Two separate DPN-linked dehydrogenases have been described which will interconvert hydroxypyruvic and D-glyceric acids. One is the crystalline glyoxylic acid reductase obtained from tobacco leaves by Zelitch (7), and the other is the purified enzyme preparation from parsley leaves reported by Stafford, Magaldi & Vennesland (8). Thus, it appears that serine may be formed by either of the two routes shown below.

$$\begin{array}{lcl}
\text{Phosphoglyceric acid} & \rightarrow & \text{D-Glyceric acid} \\
\downarrow & & \downarrow \\
\text{Phosphohydroxypyruvic acid} & \rightarrow & \text{Hydroxypyruvic acid} \\
\downarrow & & \downarrow \\
\text{Phosphoserine} & & \text{Serine} \\
\downarrow & & \\
\text{Serine} & &
\end{array}$$

Interpretation of the labeling data obtained in various laboratories studying the biosynthesis of serine from labeled pyruvate and acetate is difficult. According to Nyc & Zabin (9) pyruvate-3-C^{14} injected in weanling rats gave rise to serine with equal activities in carbon atoms 2 and 3 and alanine labeled primarily in the 3 carbon. Acetate-2-C^{14} gave rise to glycine with a C^*NH_2/C^*OOH ratio of approximately 2. These results indicate that direct conversion of the carbon chain of pyruvate to serine is not a major pathway in the rat. In a set of experiments by Kit (10) it was found that acetate-2-C^{14} in cell suspensions of Gardner Lymphosarcoma gave rise to glycine with a C^*NH_2/C^*OOH ratio of approximately 5 while the specific activity of the glutamic and aspartic acid was approximately 10 times that of the glycine. The data of Nyc & Zabin, obtained with pyruvate-3-C^{14}, can be accounted for by three steps (*a*) carboxylation of the malic enzyme, (*b*) randomization through a symmetrical C-4 dicarboxylic acid intermediate, and (*c*) decarboxylation of oxalacetic acid to phosphoenolpyruvic acid. This scheme would assume very little exchange between pyruvic acid and phosphoenolpyruvic acid. A subsequent conversion to serine via phosphoglyceric acid would give equal labeling in serine carbons 2 and 3. Predominant 2 labeling in glycine from acetate-2-C^{14} could be explained by the formation of a 2,3 labeled succinate, decarboxylation to phosphoenolpyruvic acid, and then formation of 2,3 labeled serine which could then be deformylated to give the predominant 2 labeling.

Yeast and *Escherichia coli* may possess pathways of serine synthesis

different from those found in animal cells. Wang *et al.* (11, 12) report conversion of pyruvate-2-C^{14} to glycine and serine in baker's yeast in which the C^*NH_2/C^*OOH ratios for both serine and glycine are approximately one, with very little activity in carbon 3 of serine. To explain this observation the authors visualize a scheme depending on the formation of 2,3 labeled α-ketoglutaric acid with subsequent cleavage of the resulting succinic acid to form two C_2 units, one of which is doubly labeled and gives rise to symmetrically labeled glycine. However, as shown by the fact that in the same laboratory pyruvate-2-C^{14} yielded glutamic acid with 50 per cent of its activity in the 5-carboxyl in similar anaerobic yeast (13), the 5 carboxyl of α-ketoglutarate should be more heavily labeled than the 2- and 3-carbons. Accordingly the succinate derived therefrom should be labeled twice as heavily in the carboxyl as it would be in either methylene carbon after randomization. The suggested cleavage of this succinate would then yield serine with a C^*NH_2/C^*OOH ratio significantly below 1, as is observed when acetate-1-C^{14} is the serine precursor. The observed data, in which the C^*NH_2 labeling slightly exceeds that of the C^*OOH, seems to indicate that some portion of the pyruvate-2-C^{14} was converted to serine over a direct path and a second fraction was used via glycine.

No presently known metabolic pathways appear adequate to explain the report of Abelson *et al.* (14) that the presence of unlabeled glycine had no effect on the specific activity of serine formed from uniformly labeled glucose by *E. coli* whereas, under similar circumstances, unlabeled glycine reduced the specific activity of serine formed from uniformly labeled fructose by 95 per cent.

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VII. Synthesis of Serine from Carbohydrate Precursors

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